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ABSTRACT

Poultry products are an important source of human salmonellosis, a common foodborne disease of public health concern. This thesis evaluated the relationship between feed withdrawal on *Salmonella* contamination of crops, ceca and carcasses of broilers at slaughter in Alberta.

Between November 2004 and April 2005, 30 matched crop and cecal samples, an additional 30 neck skins as well as information on flock and plant risk factors were collected from 63 flocks of broilers at slaughter. Cecal contents were first screened with *Salmonella* specific real-time PCR individually, and all cecal, crop, and neck skin samples from positive flocks were processed for *Salmonella* isolation.

The flock prevalence of *Salmonella* was 57.1% and with-in flock prevalence of *Salmonella* for positive flocks was 17.2%, 8.1% and 53.9% for ceca, crops, and skin respectively. Longer transport and waiting time in plant were identified as important risk factors for *Salmonella* contamination of broiler chickens at slaughter.

DEDICATION

I dedicate this thesis to my husband, Arbind Mainali and my daughters, Apurba, Anupa and Ayusha for their unconditional love and support.

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LIST OF ABBREVIATIONS

AARD	Alberta Agriculture and Rural Development
AFLB	Agri-Food Laboratories Branch
ACMSF	Advisory Committee on Microbiological Safety of Food
AT	Atypical
BAP	Blood Agar Plate
BPW	Buffer Peptone Water
CCDR	Canadian Communicable Disease Report
CDC	Centers for Disease Control and Prevention
CFIA	Canadian Food Inspection Agency
CHEF	Clamped Homogeneous Electric Field
CI	Confidence intervals
CISR	Canadian Integrated Surveillance Report
EDTA	Ethylenediaminetetraacetic acid
EC	European Commission
EFSA	European Food Safety Authority
FAO	Food and Agriculture Organization
FSEP	Food Safety Enhancement Program
HACCP	Hazards Analysis Critical Control Points
HLM	Hierarchical Linear Modeling
LEP	Laboratory on Enteric Pathogens
LIA	Lysine Iron Agar

MAC	MacConkey plate
MDM	Multivariate Data Matrix
NML	National Microbiology Laboratory
OIE	Office International des Epizooties
OR	Odds ratio
PCR	Polymerase Chain Reaction
PFGE	Pulse-Field Gel Electrophoresis
PHAC	Public Health Agency of Canada
RAM	Rambach
RV	Rappaport-Vassiliadis Broth
SD	Standard deviation
SPSS	Statistical Package for Social Sciences
TSI	Tripal Sugar Iron Agar
TT	Tetrathionate Broth
US	United States
USA	United States of America
USDA	United States Department of Agriculture
UT	Untypable
WHO	World Health Organization
XLT4	Xylose lysine tergitol 4

CHAPTER 1

Introduction

1.1 Background

Salmonellosis is an important infectious disease in humans and animals and is caused by various serovars of the *Salmonella* genus (Khakhria et al., 1997). In humans, it is considered one of the most frequent bacterial foodborne diseases posing a major public health threat in industrialized countries (D'Aoust, 1997). In the United States, it has been estimated that 1.4 million persons are infected with non-typhoidal *Salmonella*, resulting in 15,000 hospitalizations and around 400 deaths every year (Voetsch et al., 2004). The total cost associated with *Salmonella* infection in humans is estimated to be $2.4 billion annually in the U.S. (USDA, 2006). When additional costs are added, such as closure of slaughterhouses and processing plants, product recalls and loss of production, the true economic impact of this disease is much higher (Cohen et al., 1978). Every year, approximately 6,000 to 12,000 cases of salmonellosis are reported in Canada (Health Canada, 2007), but it is believed that this number is quite low due to underreporting or lack of reporting of most sporadic cases of salmonellosis. For each case of reported infectious gastroenteritis, there are an estimated 313 cases of infectious gastroenteritis occurring in the community (Majowicz et al., 2005). The annual incidence rate of salmonellosis in humans was most recently reported as 31.1/100,000 person-years in Canada (PHAC, 2006a).

Although, salmonellosis is most often a self-limiting disease, it can be serious or fatal in very young, elderly and immunocompromised people. *Salmonella* has been recently emerging as a food safety and environmental pathogen resistant to antibiotics

used in humans. Thus, the emergence of multi-drug resistant *Salmonella* strains are threatening to become a serious public health problem which underlines the need for surveillance and prudent use of antibiotics in both agriculture and human sectors (Acha & Szyfres, 2001; WHO, 1997).

Poultry and products of poultry are considered to be a major source of *Salmonella*-related foodborne disease in humans (Humphrey et al., 1988; Kimura et al., 2004; Tauxe, 1991). The site of *Salmonella* colonization and carriage in poultry has been extensively studied (Lightfoot, 2004; Poppe, 2000). Ceca are identified as the primary site of *Salmonella* colonization (Barrow et al., 1988; Fanelli et al., 1971; Snoeyenbos et al., 1982), while intestinal contents are considered as the primary source of *Salmonella* contamination of litter in barns, the external surfaces and feathers of broilers, and of processed carcasses after rupture of the intestinal tract during evisceration in the slaughtering plant (Corrier et al., 1999a).

The contamination of broiler carcasses with enteropathogens remains a significant problem for the broiler industry, regulatory agencies, and consumers. Efforts to reduce *Salmonella* levels in poultry have been made in Canada and the USA. The Food Safety Enhancement Program (FSEP) was developed and implemented across agri-food processed commodity groups and shell eggs in Canada to ensure the production of safe food. Further, Hazards Analysis Critical Control Points (HACCP) principles were developed to ensure necessary controls were in place to address the identified hazards (CFIA, 2007). Canadian and U.S. baseline surveys in broiler chickens at slaughter provided the current data on prevalence of *Salmonella*, and data obtained were intended to enhance the safety of poultry products (CFIA, 2000; USDA, 1996). The prevalence of

Salmonella was lower in most product categories after the implementation of HACCP (Rose et al., 1999). Further, research has shown that the reduction of microbial contamination requires the appropriate identification and evaluation of both pre-harvest and post-harvest critical control points (Corrier et al., 1999a).

Standard management practices in commercial broiler production include the removal of feed prior to transportation to slaughter and processing in order to enhance the clearance of the gastrointestinal tract to reduce contamination of poultry carcasses (May & Lott, 1990). Feed withdrawal for 8 to10 hours appeared in resulting the least amount of feces in the intestines (Wabeck, 1972). It should therefore be effective in reducing the spread of fecal contamination of *Salmonella* during transport and processing (Papa, 1991; Rigby et al., 1980a). Longer feed withdrawal periods might increase the incidence of *Salmonella* in crops (Ramirez et al., 1997) because hungry chickens start to eat litter/droppings (Corrier et al., 1999a) and the crop environment favors the growth and survival of the pathogens (Corrier et al., 1999b; Hinton et al., 2000). Therefore, crop can serve as an important source of contamination of broiler carcasses (Hargis et al., 1995). Hargis et al (1995) reported that 52% of crops were *Salmonella*-positive compared to 15% of ceca from broilers sampled at a commercial processing plant. They also reported that crops are far more likely to be ruptured during evisceration than ceca. Thus, crop appears to be a significant critical control point in the prevention of *Salmonella* contamination of carcasses (Ramirez et al., 1997).

Almost all of the evidence regarding the association between feed withdrawal and *Salmonella* prevalence in crops and ceca is from experimental studies. In this thesis, a field study is proposed to assess this relationship under industry production conditions,

which will serve as a foundation for practical recommendations. This research will also compare the *Salmonella* prevalence between crops, ceca, and carcasses to establish the extent of cross contamination between individual chickens in a flock, and relate prevalence in the intestinal tract to carcass contamination.

1.2 Research objectives

The primary objectives of this thesis were to:

1. estimate the *Salmonella* prevalence within flocks by evaluating and comparing *Salmonella* prevalence in crops, ceca, and carcasses of broiler chickens at slaughter;
2. evaluate the relationship of the *Salmonella* prevalence in crops, ceca and carcasses of broiler chickens and feed withdrawal times at slaughter; and ,
3. determine serotypes and phagetypes of *Salmonella* isolates from broiler chickens at slaughter

The null hypotheses were:

1. there is no difference between the prevalence of *Salmonella* recovered from crops, ceca and carcasses of broiler chickens;
2. there is no association between the prevalence of *Salmonella* recovered from crops, ceca and carcasses of broiler chickens and feed withdrawal times at slaughter

CHAPTER 2

Literature Review

2.1 *Salmonella* etiology and epidemiology

The various aspects of *Salmonella* epidemiology in animals and humans have been reviewed extensively elsewhere (D'Aoust, 1997; D'Aoust et al., 2001; Hohmann, 2001; Lax et al., 1995; Miller et al., 1995). Therefore, this review describes the general aspect of epidemiology of the organism including *Salmonella* infections in animals and humans.

2.1.1 Bacteriology

Salmonellae, belonging to the family of *Enterobactericeae*, are rod-shaped, gram-negative, motile (with exceptions of *Salmonella enterica* serovar Pullorum, *Salmonella enterica* serovar Gallinarum), non spore-forming, facultatively anaerobic bacteria. Salmonellae grow within a wide temperature range, from 8°C to 45°C (optimally at 37°C), and at pH of 4 to 8. A typical *Salmonella* isolate would produce acid and gas from glucose in triple sugar iron agar (TSI) or would not utilize lactose or sucrose in TSI (D'Aoust et al., 2001; Miller et al., 1995).

The bacteria can survive freezing and desiccation, and persists in suitable organic materials for weeks, months or years. *Salmonella* species are easily inactivated by heat and sunlight as well as by common phenolic, as well as chlorine and iodine-based disinfectants (Grimont et al., 2000; Guthrie, 1992; Schwartz, 1999).

The organism possesses three major antigens: O or somatic antigen; H or flagellar antigen; and Vi or capsular antigen. Somatic antigens are associated with the cell wall and are composed of lipopolysaccharide (LPS). The LPS moiety may work as an

endotoxin that is heat-stable and may act as an important virulence factor of the organism. The flagellar antigens are associated with the peritrichous flagella, are heat-labile and protein in nature. Most of the serovars of the *Salmonella* can alternatively demonstrate H antigens in two phases: phase 1 (monophasic) and phase 2 (diphasic). The exception, *S. enterica* serovar Dublin, produces single H antigen. Vi antigen is a capsular superficial antigen, overlying the O antigen, occurring only in few serovars, the most important being *Salmonella enterica* serovar Typhi and *Salmonella enterica* serovar Paratyphi (D'Aoust, 1989; D'Aoust, 1997; D'Aoust et al., 2001; Ekperigen & Nagaraja, 1998; Grimont et al., 2000).

2.1.2 Nomenclature

2.1.2.1 Antigenic typing and *Salmonella* nomenclature

An antigenic classification scheme for salmonellae based on serological properties, was first proposed by White (1926) and further developed by Kauffmann (1941), and is commonly known as the Kauffmann-White Scheme (D'Aoust et al., 2001; Popoff et al., 2000). Classification currently includes more than 2,500 serovars, based on H and O antigens (Acha & Szyfres, 2001; Grimont et al., 2000; Heyndrickx et al., 2005; Libby et al., 2004; WHO, 2005).

2.1.2.2 Alternate systems used to classify Salmonellae

Epidemiologically, *Salmonella* species can be classified according to their adaptation to animal and human hosts. Group 1 (e.g., *S.* Typhi and *S.* Paratyphi) causes enteric fever only in humans and higher primates. Group 2 causes disease in certain animals, but only infrequently in humans. However, when these strains do cause disease in humans it is often invasive and can be life threatening. *Salmonella enterica* serovar

Cholerasuis (swine) and *S.* Dublin (cattle) belong to this group. Group 3 includes the remaining strains that typically cause gastroenteritis, which is often mild and self-limiting, but can be severe in young, elderly, and the immunocompromised patients. This group includes *S. enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis, the two most important strains for salmonellosis transmitted from animals to humans (WHO, 1997; WHO, 2005).

Phagetype (PT) is determined by the ability of selected phage preparations to produce zones of clearing on agar plates previously seeded with test organisms (D'Aoust, 1989; Grimont et al., 2000). There are value-added methods for differentiating strains of *Salmonella* serotypes, first used to differentiate strains of *S.* Typhi. These methods are applied in reference laboratories including in Laboratory Foodborne Zoonoses and OIE to other serotypes of *Salmonella,* particularly those of importance to human health such as Patatyphi, Typhimurium, Enteritidis, Heidelberg, and Hadar (Bell & Kyriakides, 2002). In the case of *S.* Typhimurium, over two hundred and sixty phagetypes can be defined using 37 typing phages (Anderson et al., 1977).

Study that used antibiograms to evaluate antimicrobial resistance patterns have been used to subgroup *Salmonella* species where paper disks impregnated with standard amounts of antibiotics are placed on the surface of a Muller-Hinton agar plate previously inoculated with the test organism. Following overnight incubation, the zones of growth inhibition on the plates are measured and resistance assessed according to the laboratory standards (D'Aoust, 1989).

DNA finger printing or Pulse-Field Gel Electrophoresis (PFGE) is a molecular typing of whole genome where DNA is isolated intact and restriction enzymes are used to

generate pieces small enough to resolve by electrophoresis in agarose (USDA, 2008). Restriction endonucleases use infrequent restriction sites in a bacterial DNA (Grimont et al., 2000). The *Salmonella* isolates showing less than 95% homology during PFGE are considered to have different PFGE profiles.

Salmonella nomenclature used for this study follows the Kauffmann-White scheme defined and maintained by the WHO Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute, Paris, France (Popoff & Le Minor, 1997). The genus *Salmonella* contains two species: 1) *S. enterica,* and 2) *S. bongori. Salmonella enterica* is further divided into six subspecies which are referred to by a Roman numeral and a name: *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), and *indica* (VI) (Popoff, 2001). The individual serovars retained their names and should be indicated with an initial capitalized letter and not italicized. For example, the serovar Typhimurium belonging to *Salmonella enterica* subsp. *enterica* would be referenced as *S.* Typhimurium.

2.2 Host range

Salmonella species are distributed worldwide and have a wide range of hosts. They are normally found in the gastrointestinal tracts of mammals, reptiles, amphibians, birds, and insects (Bell & Kyriakides, 2002; Miller et al., 1995; Tauxe, 1991; Tauxe & Pavia, 1998). They are effective commensals, as well as pathogens that cause a wide spectrum of disease in human (Miller et al., 1995). They are also widely spread in the natural environment including soil and water in which they do not usually multiply but may survive for a long period. Most of the serovars pathogenic to mammals including

humans belong to *Salmonella enterica* subspecies *enterica* (i.e., subsp. I). Some serovars are host-adapted, such as humans (serovars Typhi, Paratyphi A), sheep (serovar Abortusovis), or fowl (serovar Gallinarum) (ICMSF, 1996; Lightfoot, 2004).

2.2.1 *Salmonella* infection in humans

Salmonella infections in human can lead to several clinical conditions, including enteric (typhoid) fever, uncomplicated enterocolitis, and systemic infections by non-typhoid microorganisms. Enteric fever is serious human disease associated with human host-adapted Typhi and Paratyphi strains that rarely cause disease in developed countries, but are very common in developing countries (Bell & Kyriakides, 2002; D'Aoust et al., 2001; Miller et al., 1995; Olson et al., 2001).

Non-typhoidal salmonellosis refers to disease caused by many serotypes of *Salmonella* other than *S.* Typhi. It has a wide range of clinical syndromes from self-limited disease to acute enterocolitis as well as focal infection, bacteremia, and meningitis (Tauxe & Pavia, 1998). The most frequent form of salmonellosis is self-limiting and usually does not require medical attention (D'Aoust et al., 2001). However, *Salmonella* infection can be very severe, even life threatening for younger children, the elderly, and the persons with immunocompromised health status as well as individuals with underlying disease conditions (Hohmann, 2001). Non-typhoidal strains of *Salmonella* are important causes of reportable food-borne infection, which are of considerable clinical and public health importance (Hohmann, 2001; Tauxe & Pavia, 1998). More than 95% of cases of salmonellosis are foodborne, of these accounting for approximately 30% deaths in the United States (Mead et al., 1999). Of all *Salmonella* infections, the three serovars *S.* Typhimurium, *S.* Enteritidis, and *S.* Heidelberg – are

responsible for more than 50% of foodborne illnesses in human. The other serovars that are responsible for food-borne illnesses are *S.* Thompson, *S.* Hadar, *S.* Newport, and *S.* Infantis (PHAC, 2007b).

The prevalence of salmonellosis in humans around the world depends on the water supply, waste disposal, food production, and preparation practices as well as climate. Various factors including intensive rearing of animals, increased human population, changes in the methods of production of foodstuffs, and increasing in speed of transport of food have led to a continuing increase in the incidence of foodborne illness worldwide (Lightfoot, 2004).

The annual incidence rate per 100,000 population of non-typhoidal salmonellosis in developed countries varies from 8.4 to 77.4, as illustrated in Table 2-1.

Table 2-1 Incidence of Human Salmonellosis in Various Developed Countries

Country	Year	Annual Incidence Rate Per 100,000 Population	Ref.
Australia	2005	41.2	OzFoodNet Working Group, 2006
Austria	2005	62.9	EFSA, 2006
Belgium	2005	47.1	EFSA, 2006
Canada	2005-2006	31.3	PHAC, 2006a
Denmark	2005	33	Anonymous, 2006
Finland	2005	47.3	EFSA, 2006
France	2005	9.4	EFSA, 2006
Germany	2005	63.6	EFSA, 2006
Hungary	2005	77.4	EFSA, 2006
Ireland	2005	8.4	EFSA, 2006
Italy	2005	8.6	EFSA, 2006
The Netherlands	2005	8.5	EFSA, 2006
New Zealand	2007	30.4	Anonymous, 2007
Norway	2005	32.2	EFSA, 2006
Spain	2005	14.1	EFSA, 2006
Sweden	2005	35.2	EFSA, 2006
United Kingdom	2005	21.3	EFSA, 2006
United States	2005	14.55	CDC, 2006

2.2.2 *Salmonella* transmission

As animals are the reservoir for salmonellae, any food of animal origin can be a source of infection for humans. The most common vehicles are contaminated poultry, pork, beef, eggs, milk, milk, and eggs products, as well as contaminated fruits and vegetables. Therefore, the main routes of transmission to humans are mainly between humans and from animals through the food supply and from contaminated water and the environment (Acha & Szyfres, 2001; Bell & Kyriakides, 2002). Humans can also contract infections directly from domestic animals or house pets such as dogs, cats, turtles, snakes, monkeys, hamsters, and others (Acha & Szyfres, 2001).

2.2.3 *Salmonella* surveillance

2.2.3.1 *Salmonella* surveillance – Canada

A national foodborne disease reporting system in Canada has been collecting data since it was established in 1973 (Todd, 1992). The Canadian Integrated Surveillance Report (CISR) for *Salmonella* in 1995 indicated that there were 7,307 human cases of salmonellosis reported through provincial laboratories to the National Laboratory for Bacteriology and Enteric Pathogen Database. The top three serovars, *S.* Typhimurium, *S.* Enteritidis, and *S.* Heidelberg accounted for 1,366, 964 and 670 cases of human salmonellosis, respectively (Health Canada, 1998). The CISR for *Salmonella* in 2003 reported that the number of human salmonellosis cases declined from 1996 to 1999 (6,650, 6,076, 7,149 and 5,724 cases), with same three serovars accounting for the majority of infection in 2003 (Health Canada, 2003). There was an increase in *Salmonella* infections from 5,504 in 2004 to 6,320 in 2005 after several years of decline.

In 2005, *S.* Enteritidis, *S.* Typhimurium, and *S.* Heidelberg were the most prevalent serovars, accounting for 28%, 17%, and 11% of infection, respectively (PHAC, 2007b).

2.2.3.2 *Salmonella* surveillance – Alberta

Salmonellosis became reportable in Alberta in 1959. There were 31.2, 27.1, 23.5, and 21.3 cases of human salmonellosis per 100,000 people reported to the provincial laboratory for the years 2001, 2002, 2003, and 2004, respectively (PHAC, 2006b). The three predominant serovars reported in 2004 were *S.* Enteritidis, *S.* Typhimurium, and *S.* Heidelberg, which, accounted for 23.6%, 21.8%, and 18% respectively of total cases of human salmonellosis in Alberta (PHAC, 2006b). In 2005, there were 685 cases of human salmonellosis reported. *S.* Typhimurium, *S.* Heidelberg, and *S.* Enteritidis remained the three top serovars, accounting for 18%, 14%, and 20% of the total isolates, respectively (PHAC, 2007b).

2.2.4 *Salmonella* in animals

In the U.S., Salmon and Smith first reported the isolation of "hog cholera bacillus" from diarrheic swine in 1885, later named *Salmonella* Cholerasuis (Smith, 1894).

Salmonellosis is a disease of all animals caused by many species of salmonellae and characterized clinically by one or more of the three major syndromes: septicemia, acute enteritis, or chronic enteritis. In some animal species, *Salmonella* can cause abortion in pregnant ewes and cows. Septicemia is common in young animals, and acute and chronic enteritis in older animals (The Merck Veterinary Manual, 2006;

Wray & Davies, 2000; Wray & Linklater, 2000). The clinically normal carrier animal is a serious problem in all host species and constitutes an important reservoir for human infections (The Merck Veterinary Manual, 2006).

Salmonellosis occurs worldwide and the incidence has increased with the intensification of livestock production (The Merck Veterinary Manual, 2006). Various serovars of *Salmonella* can cause disease in animals. *Salmonella* Typhimurium, *S.* Dublin, and *S.* Newport appear to be the more common serovars isolated from cattle (Ekperigen & Nagaraja, 1998; The Merck Veterinary Manual, 2006; Wray & Davies, 2000); whereas *S.* Typhimurium, *S.* Dublin, *S.* Abortusovis, and *S.* Montevideo are the most prevalent serovars in sheep (Ekperigen & Nagaraja, 1998; The Merck Veterinary Manual, 2006; Wray & Linklater, 2000). Similarly, the most common serovars that cause disease in pigs are *S.* Cholerasuis and *S.* Typhimurium (Ekperigen & Nagaraja, 1998; Fedorka-Cray et al., 2000; The Merck Veterinary Manual, 2006) and in horses are *S.* Typhimurium, *S.* Anatum, *S.* Newport, and *S.* Enteritidis (House & Smith, 2000; The Merck Veterinary Manual, 2006).

2.2.4.1 *Salmonella* infection in poultry

A wide variety of *Salmonella* serovars can infect poultry and. *S.* Typhimurium, *S.* Enteritidis, and *S.* Heidelberg are among the most common ones that have public health significance (The Merck Veterinary Manual, 2006). The infection is mostly confined to the gastrointestinal tract and the organisms are often excreted in feces of the birds (Poppe, 2000). The birds usually do not show any clinical signs of the disease with exception of some degree of mortality limited to young chicks during the first few weeks of life (The Merck Veterinary Manual, 2006).

Poultry can be infected with *Salmonella* either by horizontal or vertical transmission. Horizontal spread of *Salmonella* occurs by litter, feces, feed, water, fluff, dust, insects, and contaminated equipment. Contact with chicks of other bird species, rodents, pets, wild animals, and contaminated personnel can also contribute to horizontal transmission. Vertical transmission occurs when *Salmonella* infects the follicles in the ovaries, developing eggs in the oviduct of the infected birds, or contaminated fomites are trapped via pores of freshly laid eggs (Ekperigen & Nagaraja, 1998; Poppe, 2000).

2.3 Prevalence of *Salmonella* in poultry

Since the early 1970's, several epidemiological studies have been conducted in many countries to estimate the prevalence and identification of serovars of *Salmonella* in the poultry production cycle and retail.

2.3.1 Prevalence study during broiler chicken production

The prevalence of *Salmonella* species described in studies conducted on farm for several countries is summarized in Table 2-2. Similarly, within flock prevalence of *Salmonella* determined by studies conducted on farm, at abattoir, and at the laboratory during postmortem examination is illustrated in Table 2-3. These published reports indicate that flock prevalence of *Salmonella* varies from 0 to 100% and within flock prevalence, or sample level prevalence, can range from <0.01% to 60.9%. Hence, one should be careful and cautious while comparing the results among these studies as some of the differences might be attributed due to differences in sampling plans, sample type, and microbiological methods used to isolate the organisms. Further, some of these countries have a good *Salmonella* control program in place for entire food chain from

farm to the fork, and therefore have a low prevalence of *Salmonella* for the entire food chain.

Table 2-2 Flock Prevalence of *Salmonella* in Broiler Chickens as Determined by Studies Conducted during Production in Various Countries

Country	Year	No. of Flocks Tested	Type of Samples	Prevalence %	Reference
Austria	1998	5,029	Cloaca	3.4	EC, 1998
	2005-2006	365	Socks	5.4	EFSA, 2007
Belgium	1998	122	Feces	36.1	EC, 1998
	2005-2006	373	Socks	12.4	EFSA, 2007
Canada	1989-1990	294	Litter	75.9	Poppe et al., 1991
		290	Feed	13.4	
Canada*	2003	58	Carcass rinse	87.5	Boulianne et al., 2004
	2003	81	Ceca	50.0	Arsenault et al., 2007
Denmark	1998	4,166	Socks	6.5	EC, 1998
	1998-1999	8,911	Socks	5.5	Wedderkopp et al., 2001
	2003	1,552	Cloaca	5.0	Anonymous, 2004a
	2005-2006	295	Socks	1.6	EFSA, 2007
Finland	1998	2,856	Feces	0.7	EC, 1998
	2005-2006	360	Socks	0.1	EFSA, 2007
France	1996-1997	86	Litter swab	69.8	Rose et al., 1999
	2005-2006	381	Socks/swab	6.2	EFSA, 2007
Germany	1998	455	Socks	4.2	EC, 1998
	2005-2006	377	Socks	15.0	EFSA, 2007
Hungary	2005-2006	359	Socks	68.2	EFSA, 2007
Ireland	1998	1,732	NS	20.7	EC, 1998
	NS	40	Neck skin	55.0	White et al., 2002
	2005-2006	351	Socks	27.6	EFSA, 2007
Italy	1998	1,093	NS	3.1	EC, 1998
	2005-2006	313	Socks	28.3	EFSA, 2007
Japan	1995-1998	35	Feces	57.1	Murakami et al., 2001
Netherlands	1998	192	NS	31.8	EC, 1998
	2005-2006	362	Socks	7.5	EFSA, 2007
Norway	2005-2006	320	Socks	0.1	EFSA, 2007
Senegal	2000-2001	70	Droppings	28.6	Cardinale et al., 2004

Note: NS= Not Stated

* *Study was conducted in the province of Quebec*

Contd. Table 2-2 Flock Prevalence of *Salmonella* in Broiler Chickens as Determined by Studies Conducted during Production in Various Countries

Country	Year	No. of Flocks Tested	Type of Samples	Prevalence %	Reference
Spain	2005-2006	388	Socks	41.2	EFSA, 2007
Sweden	1998	2,935	Feces	0.03	EC, 1998
	2005-2006	291	Socks	0.0	EFSA, 2007
Thailand	1991-1992	13	Feed/litter	100	Sasipreeyajan et al., 1996
United Kingdom	2005-2006	382	Socks	8.2	EFSA, 2007
United States	NS	155	Feces	5.2	Jones et al., 1991

Note: NS = Not Stated

Table 2-3 Within Flock Prevalence of *Salmonella* in Broiler Chicken Production and Processing Determined by Studies Conducted in Various Countries

Country	Year	No. of samples	Type of sample	% Positive samples	Reference
Belgium[1]	2004	183	Carcasses	8.7	Anonymous, 2004c
Belgium[1]	2005	228	Carcasses	5.7	EFSA, 2006
Canada[2]	1975-1976	267	Litter	16.5	Hacking et al., 1978b
		412	Pelleted feed	5.6	
		35	Wood shavings	17.1	
Canada[1]	1983-1986	670	Carcass Rinse	60.9	Lammerding et al., 1988
Canada[1,3]	NS	635	Crop swab	4.3	Chambers et al., 1998
Canada[1]	1997-1998	774	Carcass Rinse	21.1	CFIA, 2000
Canada[1]	2005	NS	Cecal	18	PHAC, 2007a
Denmark[1]	2005	1,174	Broiler meat	2.3	EFSA, 2006
France[2]	NS	1,035	Litter	27.1	Lahellec & Colin, 1985
		912	Water (Drinker)	17.9	
		869	Food (Trough)	9.6	

[1] *Samples were collected at slaughter/abattoir/processing plant*
[2] *Samples were collected on farm*
[3] *Study was conducted for the Provinces of Ontario and Quebec*
Note: NS = Not stated

Contd' Table 2-3. Within Flock Prevalence of *Salmonella* in Broiler Chicken Production and Processing Determined by Studies Conducted in Various Countries

Country	Year	No. of samples	Type of sample	% Positive samples	Reference
Malaysia[1,2]	NS	104	Carcasses rinse	50.0	Rusul et al., 1996
		40	Litter	20.0	
Norway[1]	2005	6,056	Neck skin	<0.1	EFSA, 2006
Saudi Arabia[1,2]	1988-1997	114	Carcass rinse	42.9	Nakhli et al., 1999
		348	Litter	2.3	
		595	Feed	3.5	
Spain[1]	2005	203	Poultry meat	13.8	EFSA, 2006
Spain[1]	1992	90	Carcass Cloacal/ Pericloacal swab	56.7	Carraminana et al., 1997
Sweden[1]	2005	3,506	Neck skin	0.0	EFSA, 2006
Turkey[1]	NS	697	Ceca	17.0	Carli et al., 2001
United States[1,2,4]	NS	14	Carcass rinse	21.4	Jones et al., 1991
		39	Coop debris	33.0	
		155	Fecal dropping	5.2	
United States	1994-1995	1,297	Carcasses rinse	20.0	USDA, 1996
United States	1999-2000	1,225	Carcasses rinse	8.7	USDA, 2001

[1] *Samples collected at slaughter/abattoir/processing plant*
[2] *Samples collected on farm*
[4] *Samples collected from live haul trucks*

2.3.2 Prevalence in poultry retail products

Salmonella prevalence in poultry retail products among various countries is summarized in Table 2-4 below. *Salmonella* prevalence varies from as low as 2.2% in broiler meat in Belgium to 55% in chicken carcasses in Spain.

Table 2-4. Prevalence of *Salmonella* in Poultry and Poultry Products at Retails as Determined by Studies Conducted in Various Countries

Country	Year	No. of Samples	Samples Type	Prevalence %	Reference
Albania	1996-1998	461	Chicken meat	6.5	Beli et al., 2001
Belgium	1993	371	Carcasses and parts	19.4	Uyttendaele et al., 1998
	1994	473		24.1	
	1995	493		21.9	
	1996	558		36.7	
Belgium	2005	48	Broiler meat	2.2	EFSA, 2006*
Canada	2004	NS	Chicken meat	17	PHAC, 2007a
Canada	2005	NS	Chicken meat	10	PHAC, 2007a
Korea	1998	27	Carcasses	25.9	Chang, 2000
Maylaysia	NS	33	Chicken pieces	39	Arumugaswamy et al., 1995
		17	Liver	35	
		18	Gizzard	44	
Maylaysia	1995	445	Carcass rinse	35.5	Rusul et al., 1996
Spain	NS	40	Chicken carcasses	55	Capita et al., 2003
		15	Chicken parts	40	
		15	Processed chicken	40	
Sweden	2005	117	Broiler meat	6.8	EFSA, 2006*
Turkey	NS	125	Chicken parts	5.6	Guven et al., 2003
United Kingdom	1995	223	Chicken (frozen/fresh)	22.8	Plummer et al., 1995
	1998-2000	241	Whole raw chickens	25	Jorgensen et al., 2002
United States	1989	142	Chicken/parts	43	Bokanyi et al., 1990
	2001	212	Chicken	4.2	Zhao et al., 2001
	2003	251	Carcass rinse	33.9	Simmons et al., 2003

Note: NS= not stated

** Baseline survey on the prevalence of Salmonella in broiler flocks in the EU.*

2.4 Distribution of various serovars of *Salmonella* in poultry production

The dominant serovars isolated from broiler chickens during production cycles were *S.* Enteritidis and *S.* Typhimurium, ranging in prevalence from 3.1% to 81.5% and from 3.0% to 28.0%, respectively. However, in Canada, *S.* Hadar was the most prevalent serovar isolated in year 1991 and 1998. An overview of the percentage distribution of various *Salmonella* serovars during production in developed countries is summarized in Table 2-5.

Table 2-5 Percentage Distribution of Various Serovars of *Salmonella* in Poultry Production in Developed Countries

Country	No. of serotypes Isolated	*S.* Enteritidis	*S.* Typhimurium	*S.* Hadar	*S.* Heidelberg	*S.* Infantis	*S.*Agona	*S.*Mbandaka	Reference
Belgium	1443	30.0	8.0	3	-	5	5	2	EFSA, 2006
Canada	294	3.1	5.8.	33	4.8	8.8	5.1	3.7	Poppe et al., 1991
	27	-	7.4	48	11	-	3.7	7.4	Chambers et al., 1998[2]
Denmark	86	8.0	28	-	-	24	-	-	EFSA, 2006
	486	19.8	17.9	2.9	0.6	17.5	1.2	0.6	Wedderkopp et al., 2001
[3]**Finland**	17	-	12	-	-	6	-	-	EFSA, 2006
Germany	109	61	12	-	-	9	-	-	EFSA, 2006
Netherlands	1347	32	10	2	-	12	3	5	EFSA, 2006
[4]**Turkey**	119	81.5	-	-	-	-	7.6		Carli et al., 2001
[3]**United Kingdom**	694	7	3	-	-	1	1	4	EFSA, 2006

[1] Percentage of flocks in which Salmonella serovars were isolated
[2] Provincial study (Provinces of Ontario and Quebec).
[3] S. Livingstone most common serovar isolated (71% in Finland and 25% in United Kingdom).
[4] S. Sarajane was reported for the first time in chicken.

In 2005, the five most frequently isolated serovars in Canada from non-human sources were *S.* Heidelberg (22%), *S.* Typhimurium (17%), *S.* Kentucky (7%), *S.* Hadar (6%), and *S.* Enteritidis (4%) (PHAC, 2007b). Of these, *S.* Enteritidis, *S.* Typhimurium, and *S.* Heidelberg are among the most commonly reported serovars in human *Salmonella* infections (PHAC, 2007b). Further, according to Canadian abattoir surveillance in 2005, the top three serovars isolated from chickens were Heidelberg, Typhimurium, and Enteritidis, accounting for 29%, 5%, and 3.5% of the total isolates, respectively (PHAC, 2007a).

2. 5 Risk factors for *Salmonella* infections in broiler chicken and broiler chicken products

2.5.1 Risk factors for on-farm infections

Studies have been conducted in many countries to identify the potential risk factors for *Salmonella* contamination during the poultry production cycle. Prevention of *Salmonella* contamination in broiler chickens requires a detailed knowledge of the most important risk factors associated with its presence in the production system. Therefore, a better understanding of the risk factors during broiler production enables the formulation of appropriate intervention strategies to reduce *Salmonella* colonization and spread at the farm level, and cross contamination of carcasses during processing in order to provide a safe food product to consumers. This would ultimately reduce the risk of food-borne illnesses.

2.5.1.1 Breeder farm

The parent flock can be a source of *Salmonella* contamination in commercial broiler production as *Salmonella* species may be transmitted either vertically

(Bailey et al., 2001) or via contaminated eggs surfaces from breeder flocks to broiler chicks (Hacking et al., 1978b; Poppe et al., 1998). A breeder flock may also transmit more than one *Salmonella* strain to chicks (Byrd et al., 1999). Morris et al (1969) conducted a study of the dissemination of salmonellosis in a commercial broiler chicken operation and reported that serotypes of *Salmonella* identified in the broilers were traced to the breeder, from which the broilers originated, indicating a possible source of infection. Similarly, another Danish study reported the source of parent flock (*Salmonella* status of parent flocks) is a significant risk factor for *Salmonella* infection in broiler flocks (Skov et al., 1999).

2.5.1.2 Hatchery

The hatchery has been suggested as an important source of *Salmonella* infection in broiler flocks (Angen et al., 1996; Bhatia & McNabb, 1980; Skov et al., 1999). As the hatching eggs are received from various parent flocks, some of which can be contaminated and cross-contamination of chicks with *Salmonella* can occur. Therefore, larger hatcheries could be a significant risk factor associated with *Salmonella* infection in broiler flocks (Angen et al., 1996). Hence, *Salmonella* contamination of day old chicks was considered an important risk factor for *Salmonella* contamination of the flocks (Hacking et al., 1978b; Rose et al., 1999).

2.5.1.3 Preceding infection of the flock

Salmonella infection of broilers may occur as early as the day of placement if there is residual contamination from the preceding flock (Angen et al., 1996; Cardinale et al., 2004; Hacking et al., 1978b; Lahellec et al., 1986; Rose et al., 1999). If there is inadequate cleaning and sanitizing of the farm, there is more likelihood of

transmitting infection to new birds. This is an important source of contamination. Contaminated litter was the major problem in preventing *Salmonella* free carcasses (Hacking et al., 1978b).

2.5.1.4 Farm size/number of barns in farm

The size of the farm and the number of barns in a farm are considered important risk factors for *Salmonella* contamination of broiler flocks. Danish studies (Angen et al., 1996; Skov et al., 1999) have reported that when there are more than three poultry barns on the farm, the risk of contamination of broiler flocks with *Salmonella* increased significantly. In addition, flock size, densities, and the conditions under which a bird lives may influence the *Salmonella* status of a flock (FAO, 2005a).

2.5.1.5 Host susceptibility/severity of challenge

Within flock prevalence is very likely to vary from flock to flock depending upon a number of host factors including maternal flock *Salmonella* status (Corkish et al., 1994), virulence of *Salmonella* strain (FAO, 2005a; Nogrady et al., 2003), and breed/genetics of the birds (Bailey, 1988; Barrow et al., 2004; Berthelot et al., 1998). In addition, age and inoculum are important factors for *Salmonella* colonization in chickens, with younger birds being more susceptible and higher inoculum size being more infectious (Bailey, 1988). Morris et al (1969) conducted a study of the dissemination of salmonellosis in a broiler commercial chicken operation and reported that the infection rate in broiler chickens is reduced with age: 76% at 3 weeks of age compare to 10% at 9 weeks of age. Prevalence remains at same level until breeder chickens reached maturity with prevalence increasing to 42% (Morris et al., 1969). Similarly, another study by Sadler et al (1969) to evaluate the influence of age and

inoculum level on shedding pattern of *S.* Typhimurium found that the level of intestinal infection indicated by fecal shedding of viable *Salmonella* was correlated with bird's age and the number of organisms inoculated. The authors further reported that 10^2 organisms were required to induce detectable infection in all the 2-day-old chicks, 2/3 of 1- and 2-week-old birds, and none of the 4- and 8-week-old birds. In addition, U.S. studies by Dougherty (1976) have found that the infection rate was influenced by age with 37.5% of the chicks infected when placed in the poultry house and subsequently prevalence reduced by the 3rd or 4th week eventually dropping to 2.5% by the time of processing.

2.5.1.6 Farm management/hygiene

Management of the farm and hygienic procedures applied to the farm may influence the *Salmonella* colonization of broilers. Several factors such as level of stress within the broiler house and bird (Bailey, 1988; FAO, 2005a), as well as co-morbidity with other avian diseases may increase the susceptibility of the chickens to *Salmonella* colonization (Arakawa et al., 1992; Bailey, 1988; FAO, 2005a; Tellez & Kogut, 1994). In addition, inadequate disinfection of the broiler house, presence of rodents (Rose et al., 2000), as well as frequent personnel visits would increase the *Salmonella* incidence in the farm (Cardinale et al., 2004).

2.5.1.7 Feed

Feed contaminated with *Salmonella* species has been reported as a well-known risk factor for flock contamination (Corry et al., 2002; Hacking et al., 1978a; Morris et al., 1969; Simmons & Byrnes, 1972). Therefore, feed mills must follow adequate hygienic and processing controls to reduce the risk of contamination (Corry et al., 2002; Davies et al., 2001; Hacking et al., 1978a; Morris et al., 1969;

Rose et al., 1999; Simmons & Byrnes, 1972; Veldman et al., 1995). French studies (Rose et al., 1999) reported that feed type (i.e., pellet vs. meal) provided in the beginning of the grow period could be a risk factor for *Salmonella* contamination of the flock, whereas another study considered pelleted feed as a source of infection for the flock (Hacking et al., 1978a).

2.5.1.8 Season/environmental temperature

Season of the year was considered to be an important factor, for *Salmonella* infection with the highest risk for *Salmonella* infection of the flock occurring in the autumn, between September and November (Angen et al., 1996; Skov et al., 1999). This could be due to problems with proper cleaning, disinfection and drying during the cold season. Further, when there is a differential variation in internal and external temperature, there may be increased risk of condensed water, favouring growth conditions for *S.* enterica, and consequently the risk of persistence once introduced (Angen et al., 1996). Similarly, Soerjadi-Liem and Cumming (1984) demonstrated a higher prevalence of *Salmonella* infection in Australian flocks during cold and wet season. Stress associated with lower environmental temperature can potentially increase fecal excretion, leading to *Salmonella* excretion in *Salmonella*-positive birds (ACMSF, 1996).

2.5.2 Risk factors for carcass contamination

2.5.2.1 Transport truck/crates

The plastic crates that are used to transport birds from farm to processing can be a potential risk factor for contaminating birds. Rigby et al (1980a) studied the incidence and sources of *Salmonella* infection in a broiler flock and found that 86.6% of plastic crates used to transport the flocks were contaminated with *Salmonella* species before the

birds were loaded, whereas 73.5% of crates sampled after washing yielded *Salmonella* species. Further, carcasses from an uninfected commercially processed broiler flock were found to be positive for *Salmonella* after arrival in the processing plant though intestinal and rinsing cultures before transport failed to yield *Salmonella* species (Rigby et al., 1980b). This indicates that plastic transport crates could be a likely source of contamination in an uninfected commercially processed broiler flocks. Therefore, improved hygiene management during transport of broilers could significantly reduce the risk of *Salmonella* contamination in poultry meat (Corry et al., 2002; Heyndrickx et al., 2002).

2.5.2.2 Cross contamination in plant

Several studies have indicated that cross contamination at the processing plant could be a risk factor for *Salmonella* contamination of the carcasses (Dougherty, 1976; Lillard, 1990; Morris & Wells, 1970). Therefore, improved hygiene management in slaughterhouses could significantly reduce the risk of *Salmonella* contamination of poultry meat (Heyndrickx et al., 2002). Washing procedures within the plants would reduce *Salmonella* contamination in a poultry processing plant. However recontamination was possible at least during evisceration and chilling, suggesting the spread of salmonellae from flock to flock during the serial processing of the flocks (Morris & Wells, 1970). Various serotypes found after processing could be indicative of in-plant contamination, presumably due to previously processed flocks (Dougherty 1969).

Further, the *Salmonella* incidence for incoming birds was 19% compared to 37% for chilled carcasses (Lillard, 1990), the incidence rate increased from 30% from

incoming birds to 60% in air-chilled carcasses and 80% in cold-stored livers (Carraminana et al., 1997), providing evidence of cross-contamination in broiler processing plants.

2.5.3 Risk factor proposed for this study

2.5.3.1 Feed withdrawal time

The total feed withdrawal time refers to the combination of the feed withdrawal time on farm, duration of transport to processing plant and the waiting period before birds were processed. Hence, the stress of transport associated with factors such as vehicle conditions, length of journey, temperature, and road conditions, could increase fecal excretion, leading to *Salmonella* excretion in *Salmonella*-positive birds and the possibility of cross-contamination during transportation (ACMSF, 1996).

Various studies have suggested feed withdrawal time as a potential risk factor for *Salmonella* contamination of crops and ceca of broiler chickens (Corrier et al., 1999a; Hargis et al., 1995; Ramirez et al., 1997). The incidence of *Salmonella* in the crops and ceca of orally-challenged market-aged broiler chickens was consistently higher, ranging from 2.8 to 7.3 times for crop and 1.4 to 2.1 times for ceca, following 18 hours of feed withdrawal compared to full-fed broilers (Ramirez et al., 1997). Further, in a subsequent experiment with 8 hours of feed withdrawal at commercial broiler house, the incidence of *Salmonella* was significantly higher in crops following feed withdrawal compared to samples taken immediately prior to feed withdrawal (36/100 compared to 19/100), suggesting feed withdrawal might be a potential risk factor for *Salmonella* contamination in crop (Ramirez et al., 1997). Similarly, the incidence of *Salmonella* in crop contents of commercial broilers increased as much as five times (1.9% before and 10.0% after),

whereas no significant difference was observed in the incidence of *Salmonella* in ceca during pre-slaughter feed withdrawal (Corrier et al., 1999a). Further, overall *Salmonella* contamination of 7 weeks-old commercial broiler chickens at processing plant with pre-slaughter feed withdrawal time ranging from 11 to 13.5 hours was 52% for crops compared to 14.6% for ceca. In addition, crops were 86 times more likely to rupture than ceca during processing, suggesting crops might serve as a source of carcass contamination with *Salmonella* at processing plant (Hargis et al., 1995; Ramirez et al., 1997).

It has been suggested that the increased recovery of *Salmonella* from the crops of the birds before (3.3%) and after (12.6%) 8 hours of feed removal (Corrier et al., 1999a), and the subsequent increase in the incidence of crop contamination, indicated that feed deprivation influenced crop conditions (Corrier et al., 1999b) or creates physical, chemical and microbiological changes in the crops of broiler chickens which may either decrease the natural resistance of the birds to crop colonization by *Enterobactericeae* including *Salmonella* species (Hinton et al., 2000), or increase the survival of *Salmonella* in the crops (Corrier et al., 1999b). In addition to increased exposure to *Salmonella* resulting from the ingestion of contaminated litter (Corrier et al., 1999a), the ingested bacteria are exposed to a crop environment that contains a reduced concentration of lactic acid, less acidity is therefore more compatible to *Salmonella* survival (Corrier et al., 1999b).

2.6 Poultry production in Alberta

The poultry industry in Alberta operates under a quota system, with approximately 560 quota-holding producers (AARD, 2007). There are approximately 310-registered broiler chicken producers with an annual production of 85,805,000 kg of chickens (eviscerated weight) in 2006 (Chickens Farmers of Canada, 2007). Every commercial broiler producer must be registered with the Alberta Chicken Producers marketing organization, and the quota that the producer owns determines the number of chickens that they are permitted, and required, to produce and sell annually. Typically, Alberta broiler chicken farms have an average of 15,000 to 20,000 chickens (range 5,000 to 200,000), kept in several barns, each containing from 5,000 to 10,000 chickens. Chickens are kept as a large, single group in each barn, on straw bedding on concrete, or earthern floors. Farms might have from one to 20 barns on the property. Typically, a complete pellet or crumble ration is formulated by feed mills or on farm and provided to chickens ad libitum by automated feeding troughs. They receive water through automated drinking systems that carry water in closed lines and deliver it via pressure-operated valves or open bell-shaped water bowls.

Registered hatcheries in Alberta supply one-day- old chicks to the farms and may fill some or all of the barns on a farm at one time. The majority of hatcheries in Alberta receives eggs from broiler breeders in Alberta (90%). Approximately, 9.3% of the eggs would be imported from U.S. broiler breeders and 1% from Saskatchewan (B. Smook, personal communication, December 2007).

Broiler farms operate on an 8-week cycle, so that chickens are fed for 37 to 43 days. Chickens of that age from all barns are then trucked to a slaughter facility. Barns

are vacant for about 2 weeks to allow for cleaning before the next groups of chicks are brought in. The bedding is typically completely removed between flocks, but the degree to which floors, equipment and walls are washed varies considerably among farms.

CHAPTER 3

Materials and Methods

3.1 Selection of flocks and study population

This study was conducted at a single slaughter plant in Alberta using a 2-stage sampling procedure. The study was conducted during the winter from November 1, 2004 to April 31, 2005.

3.1.1 Selection of birds

For this study, birds in each flock were selected by using a systematic random sampling plan with every 5th bird in the processing line samples until 30 birds were chosen. This sample size permitted detection of at least one positive bird in a flock size of 10,000 birds carrying a 10% prevalence of *Salmonella* with 95% confidence (S. McEwen, personal communication, October 2004) within flock (Win Episcope 2.0). Flock is defined as group of birds of same age and raised by the same producer and processed on the same day.

3.1.2 Sample size

The number of flocks chosen for the study was based on comparison of proportions between two samples; crop and cecum (Donner & Klar, 2000).

$$N = \frac{(Z_{a/2} + Z_B)^2[P_1(1-P_2) + P_2(1-P_2)][1+(M-1)p]}{(P_1-P_2)^2}$$

P_1 = proportion in one sample (crop)

P_2 = proportion in other sample (cecum)

$P_1 - P_2$ = "0.1 - 0.2" – clinically important difference that we would like to find

Z_B = 0.84 (Power of study 0.8)

$Z_{\alpha/2}$ = 1.96 (5% Level of significance)

M = number of birds within flock

p= 0.03 (within flock correlation).

This value will allow us to adjust for clustering of the birds within a flock as they would be raised and housed in a similar way and therefore will be highly correlated.

Given these values and solving for N, we arrive at:

$$N= \underline{(1.96+0.84)^2 *((0.1*0.9) + (0.2*0.8))*(1+(30-1)*0.03)}/(0.1-0.2)^2$$

= 367

In order to detect at least 10% difference in prevalence of *Salmonella* between crops and ceca in a flock, which has intra-correlation coefficient of 0.03 with 80% power and 5% level of significance, a sample size of 367 birds each, was needed for crops and ceca. A sample of 30 birds was chosen from each of the 36 flocks.

3.2 Data collection

3.2.1 Flock data

Flock data was collected through a questionnaire completed by a plant employee and later verified by the Alberta Agriculture and Rural Development (AARD) technician. The questionnaire (Appendix 1) consisted of 2 major parts: General flock management information, and specific information from the plant. To improve the quality of data provided by plant personnel, the questionnaire was designed to be similar to the Canadian Food Inspection Agency (CFIA) *Flock Information Reporting Sheet* (Appendix 2) provided by producers. Flock level information included the age of the birds, sex, size of flocks, number of barns, any disease outbreak during grow-out period, and any

medication used. Plant information included number of birds per lot, order of kill, and condemnation rates. Feed withdrawal information included the time feeders were raised, transport time, waiting time at the plant, and the exact kill time. The questionnaire was pilot tested on two representative flocks prior to the initiation of the study. Names and addresses of the producers were not collected to maintain confidentiality for producers and the plant, but a unique identifier was assigned for each participating producer.

3.2.2 Sample collection

Due to time and labor constraints for the sample processing at the Agri-Food Laboratories, the sampling days were restricted to Monday, Tuesday, and Wednesday of each week. In order to adjust for an early kill schedule in the plant, sampling times were scheduled for 6 am, 7 am, 8 am, and 10 am. The sampling schedule was alternated on a week-by-week basis in order to make it as random as possible.

Two sampling stations were set up on the evisceration floor with the first station located immediately after venting and before evisceration, and the second station located after the final wash of the carcasses before entering the immersion chiller. Matched crop and cecal samples were collected from the 30 randomly chosen broilers in each flock using manual evisceration. These samples were placed in color-coded pre-labeled *Whirlpac*® (Nasco, Fort Atkinson, WI) bags. Red markers were used to label cecal samples and blue markers were used to label crop samples. Care was taken to prevent cross contamination between samples and birds by using separate surgical gloves for each bird. Further, numbers on the labeled bags for crops and ceca were continuously checked to make sure that matching indicators were included in the labels. The neck skin

(10-20 g) of an additional 30 birds were randomly collected from the same flock before they entered the chiller and placed in to pre-labeled *Whirlpac*® (Nasco, Fort Atkinson, WI) bags. In order to avoid cross contamination between samples, separate surgical gloves were used for each sample collection.

To have less than an hour variation in feed withdrawal time within a flock, sampling time was set for a maximum of one hour even though kill time was frequently longer than an hour. After the samples were collected in individual sterile containers, they were transported immediately to the laboratory in coolers with ice packs for qualitative *Salmonella* isolation.

3.3 Sample analysis

All bacterial isolation from crops, ceca and neck skins was performed according to Health Canada's protocol (D'Aoust & Purvis, 1998) with modification at the Agri-Food Laboratories Branch (AFLB) Laboratory, Food Safety Division of AARD in Edmonton. All samples were delivered to AFLB Laboratory on ice immediately after collection and processed within 12 hours of receiving samples at the laboratory.

3.3.1 Screening of positive flocks

All 1890 cecal samples from 63 flocks were screened by real-time PCR (Polymerase Chain Reaction) (Bohaychuk et al., 2007). The isolation of *Salmonella* from ceca is considered the most reliable indication that the bird is infected (Rigby & Pettit, 1978). The flock was considered positive if any of the cecal samples out of 30 tested positive using real-time PCR. Only crop, cecal, and neck skin samples from those positive flocks were processed further.

3.3.2 *Salmonella* isolation and identification

Appendix 3 provides the detailed description of isolation and identification procedures for the *Salmonella* species. Presumptive *Salmonella* isolates were analyzed by Pulse-Field Gel Electrophoresis (PFGE)(CDC, 2004) within a flock to check for genetic similarity.

3.3.3 Pulse-Field Gel Electrophoresis (PFGE)

All isolates of *Salmonella* were fingerprinted by Standardized Laboratory Protocol for Molecular Subtyping of *Escherichia coli* O157:H7 by PFGE, (Centers for Disease Control and Prevention, Atlanta, GA) with modifications outlined in Appendix 4.

The *Salmonella* isolates with less than 95% homology during PFGE were considered to have different PFGE profiles. A representative isolate from each different PFGE profile found in each flock was forwarded to the Public Health Agency of Canada (PHAC) in Guelph for confirmation by serotyping.

3.3.4 Serotyping and phagetyping of *Salmonella* isolates

Serotyping and phagetyping of *Salmonella* isolates were performed at the Office International des Épizooties (OIÉ) Reference Laboratory for Salmonellosis, Laboratory for Foodborne Zoonoses, Public Health Agency of Canada (PHAC), Guelph, Ontario, Canada.

The slide agglutination procedure was used to determine O or somatic antigens of the *Salmonella* isolates (Ewing, 1986). The H or flagellar antigens were identified using a microtechnique (Shipp & Rowe, 1980), which employed microtitre plates. The antigenic formulae of Le Minor and Popoff (2001) were used to name the serovars.

For phagetyping of *Salmonella* isolates, the standard technique described by Anderson and Williams (1956) was employed. *Salmonella* Enteritidis isolates were phagetyped with typing phages obtained from the Laboratory of Enteric Pathogens (LEP), Health Protection Agency, Centre for Infections, London, United Kingdom (Ward et al. 1987) via the National Microbiology Laboratory (NML), PHAC, Winnipeg, Manitoba, Canada. The phagetyping scheme and phages for *Salmonella* Typhimurium, developed by Callow (1959) and further extended by Anderson (1964) and Anderson et al (1977) were obtained from the LEP via the NML. The *Salmonella* Heidelberg phagetyping scheme and phages were supplied by the NML (Demczuk et al., 2003). *Salmonella* isolates that reacted with the phages but did not conform to any recognized phagetype were considered atypical (AT) and the isolates that did not react with any of the typing phages were called untypable (UT).

3.4 Data and statistical analysis

Data were entered and merged in Excel spreadsheets (Microsoft Corporation, Redmond, Washington, USA) and subsequently verified for accuracy by checking each entry with the original hard copies. Data were processed by SPSS 13 and further analyzed by Hierarchical linear modeling (HLM) 6.0 to account for the clustering effect within flocks.

Total feed withdrawal time was calculated by subtracting the date and time feeders were raised on the farm from the date and time the birds were killed at the slaughter plant. Further, total feed withdrawal time calculation was verified by adding

feed withdrawal time on farm, transport time and waiting time at the plant prior to slaughter.

Three datasets were created for crop, ceca and skin in SPSS. For each dataset, final results from each flock were considered as outcomes. Descriptive statistics including *Salmonella* prevalence and 95% confidence intervals (CI) for each crop, cecal, and skin samples of broilers were calculated. In addition, descriptive statistics for *Salmonella* isolates including serotypes and phagetypes were explored.

Box and Whisker plot analysis was used to examine the distribution of total feed withdrawal time; on farm feed withdrawal time, transport time and waiting time at the plant. The information regarding on farm characteristics including age, mortality percentage, and number of barns were missing for several flocks, as some producers did not include these characteristics in the flock information sheets, and therefore these variables were not considered for the final analysis. Descriptive statistics including mean, range, and standard deviation (SD) were obtained in order to describe the explanatory variables for the *Salmonella* contamination level.

In addition, significant differences in the means of the various factors for positive and negative samples were evaluated. Further, the significant difference in *Salmonella* prevalence among sampling days of the week and sampling month of the year were evaluated by using two by two tables with chi-square statistics.

Before data was processed by HLM, level I, which included bird level data (outcome) and level II which included flock level data (explanatory variables) were created in SPSS. After that, Multivariate Data Matrix (MDM) files were then created for each dataset.

Level 1 Model

$Prob(Outcome_{ij} = 1/B_j) = \varphi_{ij}$

$Log(\varphi_{ij}/(1-\varphi_{ij})) = B_{oj}$

Level 2 Model

$B_{oj} = \gamma_{oo} + u_{oj}$

Where

φij= Predicted value or probability of success

$Log(\varphi_{ij}/(1-\varphi_{ij}))$= Log odds of success

B_{oj} = Expected outcome

γ_{oo} = Average intercept across Level 2 units

uoj= Unique increment to the intercept associated with Level 2 unit j

For each data file, a two-stage procedure was used to select the important variables associated with status of the flock by HLM for discrete outcome by using Bernoulli distribution. In the first stage, univariate analysis was performed using HLM to relate *Salmonella* infection of the flock to each explanatory variable. Only factors associated up to 30% level of significance (p- value = 0.3) with *Salmonella* infection of the flock as well as the factors of interest were considered for the multivariate analysis. The second stage involved multivariate analysis by applying stepwise procedures for each factor. Total feed withdrawal time was highly correlated with feed withdrawal time on farm, transport time and wait time. Therefore, they were entered separately with other factors.

In both univariate and multivariate analysis, the population average model was used rather than the unit specific model. The population average model is more useful in

addressing epidemiology type assessment of exposure effects through outcome experience in larger groups of subjects. The main objective was to determine the effect of feed withdrawal time on *Salmonella* contamination at the flock level and not at the bird level (Hosmer & Lemeshow, 2000).

McNemar test was used to test the association between the results from crop and cecal samples, and Kappa statistic was used to test the agreement between the results from the two samples.

CHAPTER 4

Results

4.1 Descriptive results

4.1.1 Results from *Salmonella* cultures

In total, 30 samples each were collected from 63 flocks in the study. Of the 63 flocks, 36 had at least one sample positive for *Salmonella* species detected by real-time Polymerase Chain Reaction (PCR) resulting in flock prevalence of 57.1% (95% CI: 44.9%, 69.3%). From the 1890 cecal samples of 63 flocks, *Salmonella* species were isolated by culture from 186 samples, resulting in a sample level prevalence of 9.8% (95% CI: 8.4%, 11.1%). As shown in Table 4-1, among the 1080 cecal samples from the 36 positive flocks, *Salmonella* species were isolated from 186 samples resulting in a sample-level prevalence of 17.2% (95% CI: 14.8%, 19.4%). Out of 1080 crop and skin samples from 36 positive flocks, *Salmonella* species were isolated from 88 and 582 samples, resulting in sample-level prevalence of 8.1% (95% CI: 6.5%, 9.7%) and 53.9% (95% CI: 50.8%, 56.8%) for crop and skin respectively (Table 4-1).

Table 4-1 Distribution of *Salmonella* Positive Broiler Chickens by Sample Types

Sample* Type	*Salmonella* Positive #	*Salmonella* Prevalence (95% confidence interval) for Positive Flocks
Crop	88	8.1 (6.5,9.7)
Cecum	186	17.2 (14.8,19.2)
Skin	582	53.8 (50.8,56.8)

** Total number of samples: 1080*

Table 4-2 Number and Percentage of the Flocks with Various Range of Contamination Level of *Salmonella* for Different Sample Types

Sample type	Flocks with 1-10 positive samples		Flocks with 11-20 positive samples		Flocks with 21-30 positive samples	
	#	%	#	%	#	%
Crop	34	94.4	2	5.6	0	0
Cecum	33	91.7	2	5.6	1	2.8
Skin	13	36.1	10	27.8	13	36.1

As shown in Table 4-2, most of the flocks have within flock *Salmonella* contamination for crop and cecal samples ranging from 1 to 10 samples (94% for crop and 91% for cecal samples), whereas none had between 21 and 30 positive crop samples and only 1 flock had between 21 and 30 cecal positive samples. In addition, skin samples from within a flock had *Salmonella* contamination ranging from 1 to 10 samples for 13 flocks, 11 to 20 samples for 10 flocks, and 21 to 30 samples for 13 flocks.

Table 4-3 Number and Percentages of *Salmonella* Positive Broiler Chickens by Sample Type and Day of the Week for Positive Flocks

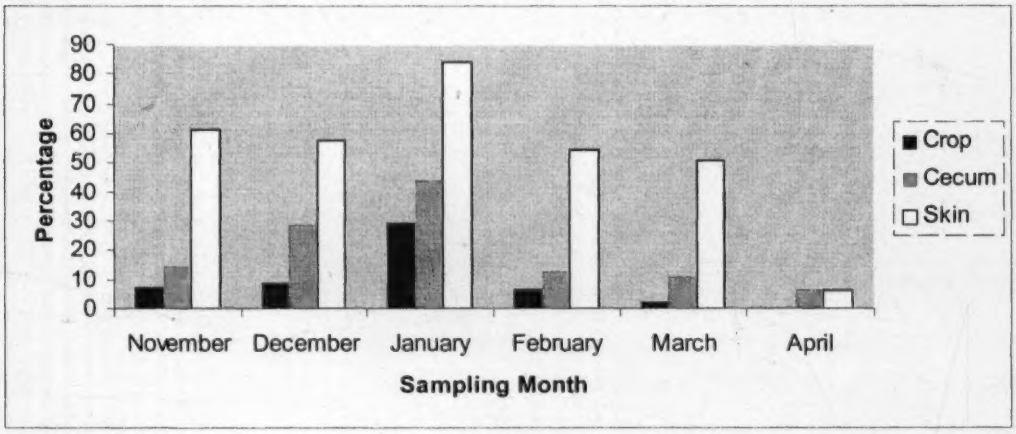
Sample type	Monday		Tuesday		Wednesday	
	#	% (N=270)	#	% (N=540)	#	% (N=270)
Crop	30	11.1	36	6.6	22	8.1
Cecal	41	15.2	101	18.7	44	16.3
Skin	126	44.6	299	53.4	157	58.1

N= total number of samples processed

The prevalence of *Salmonella* for crop, cecal, and neck skin samples is shown for the three sampling days for positive flocks in Table 4-3. There were no significant differences in *Salmonella* prevalence between sampling days of the week for crop samples ($X^2 = 4.84$, df =2, $p = 0.09$) and cecal samples ($X^2 = 1.94$, df = 2, $p = 0.38$) of

the broiler chickens at slaughter. However, the sampling day of the week seemed to influence the *Salmonella* prevalence for skin samples. The prevalence of *Salmonella* on skin was the highest on Wednesday, and was significantly different ($X^2 = 8.12$, df = 2, $p = 0.02$) compared to those on Monday and Tuesday.

Figure 4-1 Percentage Distribution of *Salmonella* Positive Broiler Chickens by Sample Type and Month of the Year for Positive Flocks



Total number of each sample in November: 300, December: 90, January: 120, February: 270, March: 180, & April: 120

The prevalence of *Salmonella* for crop, cecal, and neck skin samples for positive flocks is illustrated for the six sampling months in Figure 4-1. There were significant differences in prevalence of *Salmonella* for crops ($X^2 = 91.99$, df = 5, $p < 0.001$), ceca ($X^2 = 89.60$, df = 5, $p < 0.001$), and skin ($X^2 = 163.87$, df = 5, $p < 0.001$) between sampling months. The highest prevalence of *Salmonella* was observed in January for all three samples and the lowest was observed in April.

4.1.2 Results of serotypes and phagetypes of *Salmonella* isolates

The *Salmonella* isolates showing less than 95% homology during PFGE analysis were considered to have different PFGE profiles and were characterized by serotyping.

The serovars Heidleberg, Typhimurium, and Enteritidis were further phagetyped as these are the only phages available at Laboratory of Foodborne Zoonoses.

A total of 24 different *Salmonella* isolates were obtained from 88 *Salmonella*-positive crop samples from 36 flocks. Among the 24 *Salmonella* isolates, the most frequently isolated serovar was Hadar (66.7%), followed by Heidelberg (12.5%). The others serovars such as Blockley, 1:4,5,12:i:-(monophasic Typhimurium), and Kentucky were recovered in less than 5% of samples. The distribution of *Salmonella* serovars and phagetypes from crop samples of broilers at slaughter is described in Table 4-4.

Table 4-4 Distribution of *Salmonella* Serovars and Phagetypes from Crop Samples of Broilers at Slaughter

***Salmonella* serovars**	**Number of isolates (%) (N=24)**	**Phagetypes**	**Number of Phagetypes (%) (N=4)**
Hadar	16 (66.7)		
Heidelberg	3 (12.5)		
		AT[1]	2 (50.0)
		36	1 (25.0)
I:4,5,12:i (monophasic Typhimurium)	1 (4.2)		
		UT6[2]	1 (25.0)
Blockley	1 (4.2)		
Kentucky	1 (4.2)		
I:ROUGH-O:-:1,5	1 (4.2)		
Ohio var. 14 +	1 (4.2)		

[1] *AT : atypical*
[2] *UT : untypable*

Among 186 isolates from *Salmonella* positive cecal samples, 66 were further characterized by serotyping. Out of 66 *Salmonella* isolates, the most frequently isolated

serovars were Hadar, Heidelberg, Blockley, and Kentucky. The most common serovar was Hadar, accounting for 33.3% of the isolates, followed by Heidelberg, Blockley, and Kentucky representing 10.6%, 7.6%, and 7.6% of the total *Salmonella* isolates respectively. Of the 66 *Salmonella* isolates, 14 isolates were further phagetyped. All the serovars I:4,5,12:i:- (monophasic Typhimurium) were phagetype 191. The distribution of *Salmonella* isolates where more than one serovar were recovered from ceca of broilers at slaughter is described in Table 4-5, which also describes phagetype distribution among isolates of serovars Heidelberg, Typhimurium var. Copenhagen, as well as I:4,5,12:i:- (monophasic Typhimurium).

Table 4-5 Distribution of *Salmonella* Serovars and Phagetypes from Ceca of Broilers at Slaughter

Salmonella serovar*	No. of isolates (%) (n=66)	Phagetype	No. of Phagetypes (%) (N=14)
Hadar	22 (33.3)		
Heidelberg	7 (10.6)		
		32	2 (14.3)
		AT[a]	2 (14.3)
		18	1 (7.1)
		19	1 (7.1)
		36	1 (7.1)
Blockley	5 (7.6)		
Kentucky	5 (7.6)		
Cubana	3 (4.5)		
I: 4,5,12:i:- (monophasic Typhimurium)	3 (4.5)		
		191	3 (21.4)
I:ROUGH-O:z10:enx	3 (4.5)		
Mbandaka	3 (3.5)		
Typhimurium var. Copenhagen	3 (3.5)		
		UT2[b]	2 (14.3)
		104	1 (7.1)
I:ROUGH-O:k:1,5	2 (3.0)		
Infantis	2 (3.0)		
Thomson	2 (3.0)		

** Serovars shown if more than one is recovered and the total number of Phagetype do not add up to 14 because one of the serovar was equal one*
[a] *AT : atypical*
[b] *UT : untypable*

As illustrated in Table 4-6, of 182 isolates of *Salmonella*- positive skin samples from 36 flocks, serovar Hadar was the most frequently isolated serovar representing 39.6% of the isolates. The second most common isolated serovar was Heidelberg (14.8%), which is followed by I:4,5,12:i:-(monophasic Typhimurim), Blockley, and Kentucky, representing 7.7%, 7.1%, and 4.9% of the isolates respectively. Among Heidelberg serovars,

phagetype 19 was the most common (20.0%). Similarly for serovar I:4,5,12:i:- (monophasic Typhimurium), phagetype 191 was the most frequently isolated (6.0%).

Table 4-6 Distribution of *Salmonella* Serovars and Phagetypes from Skin of Broilers at Slaughter

Salmonella serovars*	No. of isolates (%) (n=182)	Phagetypes	No. of Phagetypes (%) (N=50)
Hadar	72 (39.6)		
Heidelberg			
		19	10 (20.0)
		32	5 (10.0)
		41	4 (8.0)
	27 (14.8)	11a	2 (4.0)
		46	2 (4.0)
		UT[1]	2 (4.0)
		18	1 (2.0)
		21	1 (2.0)
I:4,5,12:i:-(monophasic Typhimurium)	14 (7.7)		
		191	6 (12.0)
		AT[2]	5 (10.0)
		120	1 (2.0)
		104b	1 (2.0)
		UT6	1 (2.0)
Blockley	13 (7.1)		
Kentucky	9 (4.9)		
Agona	6 (3.3)		
Infantis	6 (3.3)		
I:ROUGH-O:z10:	5 (2.7)		
Typhimurium var. Copenhagen	5 (2.7)		
		104	3 (6.0)
		UT6	1 (2.0)
		UT7	1 (2.0)
Mbandaka	3 (1.6)		
Thomson	3 (1.6)		
Enteritidis	2 (1.1)		
		13	2 (4.0)
I:6,8:z10:-	2 (1.1)		

** Serovars shown if more than one is recovered and the total number of Phagetype do not add up to 50 because two of the serovars were equal one*

[1] UT : untypable

[2] AT : atypical

The serovar Hadar was the most common serovar isolated from *Salmonella* positive crop, cecal, and neck skin samples, indicating the most prevalent serovar on the farm. Similarly, Heidelberg was the second most common serovar isolated from these samples. The distribution of the first three common serovars isolated from crop, cecal, and neck skin samples is illustrated in Figure 4-2.

Figure 4-2 Distribution of Three Most Common Serovars Isolated from Crop, Cecal and Neck Skin Samples of Broiler Chickens.

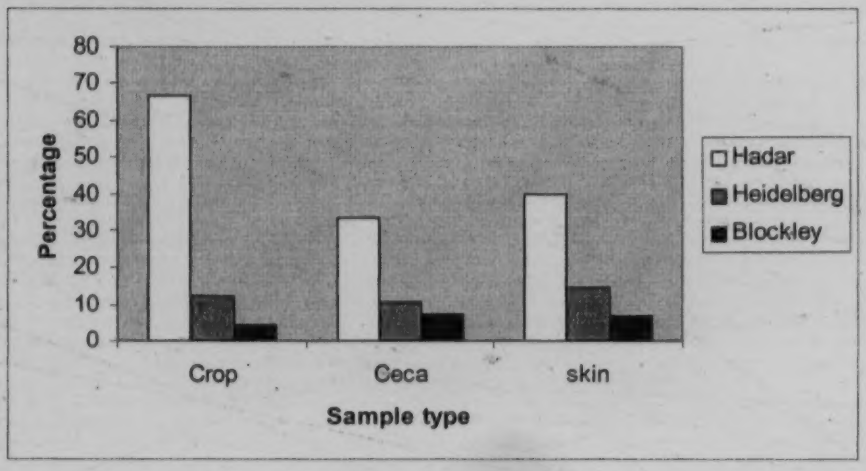


Table 4-7 shows the distribution of different factors, which could influence the *Salmonella* contamination level on crops, ceca, and carcasses of broiler chickens. The total feed withdrawal time ranged from 9.7 hours to 17.7 hours, with a mean of 12.8 hours. Similarly, flock size varied from 15,000 to 86,000 with an average of 34,953 and standard deviation of 17,310. There was a large variation in outdoor temperature, ranging from $-31^{\circ}C$ to $9.5^{\circ}C$.

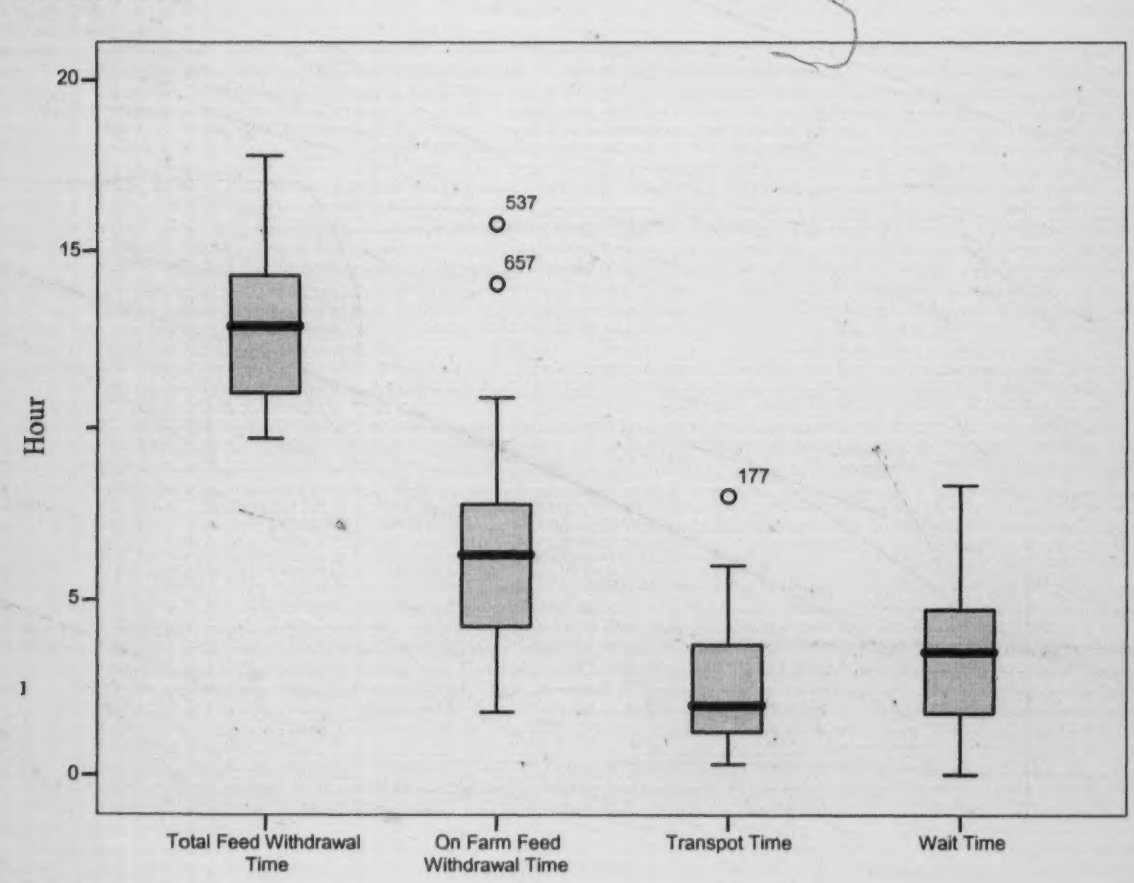
Table 4-7 Distribution of Total Feed Withdrawal, On-farm Feed Withdrawal, Transport and Wait Times, and Other Flock and Plant Characteristics

Factors*	Mean	SD	Range
Total feed withdrawal time (hr)	12.8	2.2	9.7-17.7
On-farm feed withdrawal time (hr)	6.8	3.1	1.8-15.8
Transport time (hr)	2.5	2.0	0.3-8.0
Wait time (hr)	3.4	2.1	0.0-8.3
Flock size (in 1,000)	34.95	17.31	15-86
Outdoor Temperature (°C)	-7.1	7.6	(-31.0)-9.5
Quality program (%)	0.6	0.5	0.0-1.0
Condemn percentage at plant	1.4	0.8	0.4-4.6
Kill order at plant	3.1	0.9	2.0-5.0

* *Total number of flocks: 30*

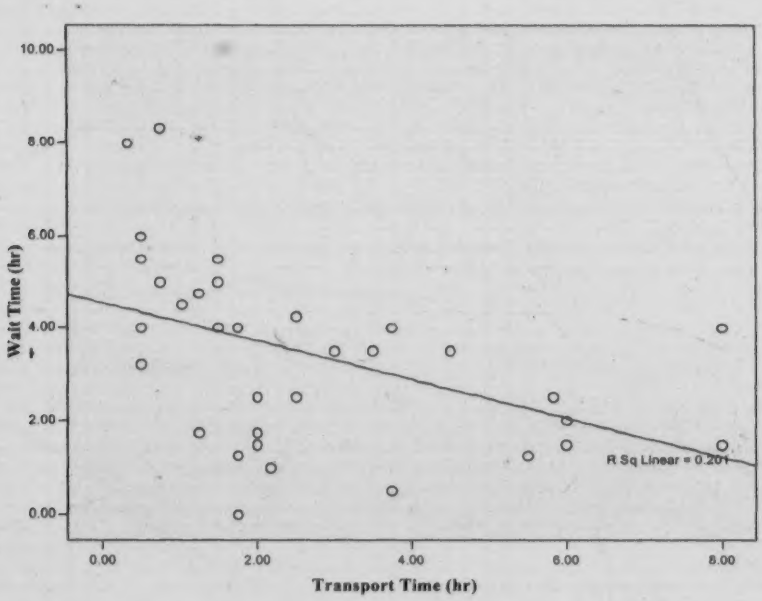
Examination of the Box and Whisker plots in Figure 4-3 revealed that the distribution of transport time is skewed to the left, indicating smaller transport times for a large number of flocks. Further the plot revealed there were two outliers for on-farm feed withdrawal time and one outlier for transport time. The distribution of on-farm feed withdrawal time and wait time were slightly skewed to the right, indicating the presence of some flocks with larger on-farm feed withdrawal and wait times.

Figure 4-3 Box & Whisker Plots Showing the Distribution of Flock and Plant Characteristics



Numbers above the top whiskers (177, 537 and 657) represent sample identification number for outliers

Figure 4-4 Scatter Plot Showing Correlation between Transport Time and Wait Time at Plant



The examination of the scatter plot between transport times and wait times at plant shows a negative relationship between these two factors- the longer the transport time, the shorter will be the wait time at plant (Pearson Correlation coefficient = 0.45, p- value = 0.008).

4.2 Analysis of crop data

4.2.1 Descriptive analysis

The distribution of total feed withdrawal time, on-farm feed withdrawal time, transport time, wait time, and other plant characteristics are shown in Table 4-8 for *Salmonella* positive and negative crop samples. There was no significant difference in the mean total feed withdrawal time between positive and negative *Salmonella* samples.

However, the mean wait time was significantly greater for positive samples in comparison to the negative samples ($p = 0.05$).

Table 4-8 Distribution of Plant and Flock Characteristics for Positive and Negative Crop Samples

Factors	*Samonella* Positive Mean (SD)	*Samonella* Negative Mean (SD)	p-value*
Total feed withdrawal time (hr)	12.9 (2.2)	12.8 (2.1)	0.99
On farm feed withdrawal time (hr)	6.3 (3.0)	6.5 (2.1)	0.73
Transport time (hr)	2.3 (2.1)	2.6 (2.1)	0.23
Wait time (hr)	4.2 (1.8)	3.4 (1.9)	0.05
Flock size (in 1,000s)	44.1 (23.7)	32.5 (15.5)	0.07
Outdoor temperature (^{0} C)	-8.8 (5.4)	-7.0 (7.8)	0.22
Kill order at plant	3.0 (1.0)	3.1 (0.9)	0.24

* *p-values were for comparison of means obtained from univariate analysis after adjusting for correlation within flocks using HLM.*

4.2.2 Univariate analysis

Table 4-9 Association Between Prevalence of *Salmonella* and Flock and Plant Characteristics from Univariate Regression Using HLM

Factors	OR (95%CI)	p-value
Total feed withdrawal time (hr)	1.0 (0.7,1.4)	0.99
On-farm feed withdrawal time (hr)	0.9 (0.8,1.1)	0.73
Transport time (hr)	0.9 (0.7,1.1)	0.23
Wait time (hr)	1.2 (1.0,1.5)	0.05
Flock size (in 1,000s)	1.0 (1.0,1.0)	0.07
Outdoor temperature (^{0} C)	0.9 (0.9,1.0)	0.22
Quality program	0.7 (0.2,2.1)	0.51
Plant condemn percentage	1.2 (0.7,1.9)	0.51
Kill order at plant	0.7 (0.4,1.2)	0.24

The results of the univariate logistic regression analysis (Table 4-9) indicated that wait time is significantly associated with the prevalence of *Salmonella*.

4.2.3 Multivariate analysis

In addition to wait time and the variables that were significant at 30%, and other factors of interest were included in the multivariate analysis to allow for potential confounding. As shown in Table 4-10, after controlling for flock size and environmental temperature, with every hour increase in wait time in plant, the risk of *Salmonella* contamination in crops of broiler chickens at slaughter would increase by 20% (p- value = 0.05).

Table 4- 10 Association between Prevalence of *Salmonella* and, Flock and Plant Characteristics from Multivariate Regression Using HLM

Factors	OR (95% CI)	p-value
Wait time (hr)	1.2 (1.0,1.4)	0.05
Flock size (in 1000s)	1.0 (1.0,1.0)	0.09
Outdoor temperature (^{0} C)	0.9 (0.9,1.0)	0.14

4.3. Analysis of cecal data

4.3.1 Descriptive analysis

The distribution of total feed withdrawal time, on-farm feed withdrawal time, transport time, wait time in plant and other flock and plant characteristics are shown in Table 4-11 for *Salmonella* positive and negative cecal samples. There was no significant difference in the mean total feed withdrawal time for positive and negative *Salmonella* samples.

Table 4-11 Distribution of Plant and Flock Characteristics for Positive and Negative Cecal Samples

Factors	*Salmonella* Positive Mean (SD)	*Salmonella* Negative Mean (SD)	p-value*
Total feed withdrawal time (hr)	13.0 (2.2)	12.8 (2.1)	0.63
On-farm feed withdrawal time (hr)	6.8 (3.0)	6.4 (2.9)	0.77
Transport time (hr)	2.2 (2.1)	2.7 (1.9)	0.23
Wait time (hr)	3.8 (1.9)	3.4 (1.9)	0.12
Flock size (in 1,000s)	38.0 (19.7)	32.5 (15.7)	0.12
Outdoor temperature (^{0} C)	- 8.8 (6.3)	-6.8 (7.8)	0.13
Kill order at plant	3.1 (0.9)	3.1 (1.0)	0.5

** p-values were for comparison of means obtained from univariate analysis after adjusting for correlation within flocks using HLM.*

4.3.2 Univariate analysis

Table 4-12 Association between Prevalence of *Salmonella* and Flock and Plant Characteristics from Univariate Regression Using HLM

Factors	OR (95% CI)	p-value
Total feed withdrawal time (hr)	1.1 (0.8,1.4)	0.63
On-farm feed withdrawal time (hr)	1.0 (0.9,1.2)	0.77
Transport time (hr)	0.9 (0.7,1.1)	0.23
Wait time (hr)	1.1 (1.0,1.3)	0.12
Flock size (in 1,000s)	1.0 (1.0,1.0)	0.12
Outdoor temperature (^{0} C)	0.9 (0.9,1.0)	0.14
Quality program	0.8 (0.3,1.9)	0.68
Condemn percentage at plant	1.0 (0.7,1.5)	0.99
Kill order at plant	0.8 (0.6,1.3)	0.5

The results from univariate analysis (Table 4-12) indicated that none of the factors were significantly associated with the prevalence of *Salmonella* in ceca of broiler chickens.

4.3.3 Multivariate analysis

The variables that were significant at 30% level and other factors of interest were included in the multivariate analysis to account for potential confounding. As indicated in Table 4-13, after controlling for transport time, on-farm feed withdrawal time, flock size, and outdoor temperature, every hour increase in wait time in plant would increase the risk of *Salmonella* contamination in ceca of broiler chickens at slaughter by 40%. Similarly, with each degree decrease in outdoor temperature, the risk of *Salmonella* contamination in ceca would increase by 10% after controlling for other factors. The association between prevalence of *Salmonella* and flock and plant characteristics from multivariate regression is shown in Table 4-13.

Table 4- 13 Association between Prevalence of *Salmonella* and Flock and Plant Characteristics from Multivariate Regression Using HLM

Factors	OR (95% CI)	p-value
Transport time (hr)	1.2 (1.0,1.6)	0.09
Wait time (hr)	1.4 (1.1,1.9)	0.03
On-farm feed withdrawal time (hr)	1.1 (0.9,1.1)	0.18
Flock size (in 1,000s)	1.0 (1.0,1.0)	0.34
Outdoor temperature (^{0} C)	0.9 (0.9,1.0)	0.03

4.4. Analysis of skin data

4.4.1 Descriptive analysis

The distribution of total feed withdrawal time, on-farm feed withdrawal time, transport time, wait time and other plant characteristics are shown in Table 4-14 for *Salmonella* positive and negative skin samples.

For total feed withdrawal time, there was no significant difference in comparison of means for positive and negative samples (p- value 0.25). However for size of flock, there was a significant difference (p-value 0.01) for positive and negative samples.

Table 4-14 The Distribution of Plant and Flock Characteristics for Positive and Negative Skin Samples

Factors	Outcome Positive Mean (SD)	Outcome Negative Mean (SD)	p-value*
Total feed withdrawal time (hr)	13.2 (2.1)	12.3 (2.0)	0.25
On-farm feed withdrawal time (hr)	6.7 (3.2)	6.3 (2.4)	0.35
Transport time (hr)	2.9 (2.2)	2.3 (1.8)	0.26
Wait time (hr)	3.2 (1.9)	3.6 (1.9)	0.53
Flock size (in 1,000s)	36.5 (19.8)	29.8 (10.6)	0.01
Outdoor temperature (^{0} C)	-7.3 (6.0)	-7.0 (9.2)	0.66
Quality program	60.9%	65.1%	0.71
Condemn percentage at plant	1.6 (1.2)	1.5 (1.3)	0.67
Kill order at plant	3.2 (1.0)	2.9 (0.8)	0.92

**p-values were for comparison of means obtained from univariate analysis after adjusting for correlation within flocks using HLM.*

4.4.2 Univariate analysis

The results from univariate analysis (Table 4-15) indicated that flock size was highly significant (p-value = 0.01) in association of *Salmonella* prevalence. Association between prevalence of *Salmonella* and flock and plant characteristics from univariate regression is shown in Table 4-15.

Table 4-15 Association between Prevalence of *Salmonella* and Flock and Plant Characteristics from Univariate Regression Using HLM

Factors	OR (95% CI)	p-value
Total Feed Withdrawal time (hr)	1.2 (0.9,1.5)	0.25
On-farm feed withdrawal time (hr)	1.1 (0.9,1.2)	0.35
Transport time (hr)	1.1 (0.9,1.4)	0.26
Wait time (hr)	0.9 (0.7,1.2)	0.53
Flock size (1,000)	1.0 (1.0,1.0)	0.01
Outdoor temperature (^{0} C)	0.9 (0.9,1.1)	0.66
Quality program	1.2 (0.4,3.3)	0.71
Condemn percentage at plant	1.1 (0.7,1.7)	0.67
Kill order at plant	1.0 (0.6,1.7)	0.92

4.4.3 Multivariate analysis

In addition to flock size, the other factors of interest such as total feed withdrawal time, on-farm feed withdrawal time, transport time and wait time at plant were included in the multivariate analysis. After controlling for wait time, on-farm feed withdrawal time, and flock size, transport time was significant for increasing the risk of *Salmonella* contamination in skin of broiler chickens (p-value = 0.04). Similarly, every fold increase in flock size of one thousand would increase significantly the risk of *Salmonella* contamination of the skin of broiler chickens after controlling for other factors (p-value =0.02). Association between prevalence of *Salmonella* and flock and plant characteristics from multivariate regression is illustrated in Table 4-16.

Table 4- 16 Association between Prevalence of *Salmonella* and Flock and Plant Characteristics from Multivariate Regression Using HLM

Factors	OR (95% CI)	p-value
Transport time (hr)	1.5 (1.0,2.2)	0.04
Wait time (hr)	1.2 (0.8,1.9)	0.35
On-farm feed withdrawal time (hr)	1.1 (0.9,1.5)	0.28
Flock size (in 1,000s)	1.0 (1.0,1.0)	0.02

4.5 Analysis of matched data from crop and cecal samples

The test of association of *Salmonella* prevalence between crop and cecal samples seemed significant (p-value<0.001). However, the measurement of agreement between two tests was only 25.7% (kappa value = 0.257). The percentage distribution of *Salmonella* negative and positive results for crop and cecal samples of broilers at slaughter is shown in Table 4-17.

Table 4-17 Percentage Distribution of *Salmonella* Negative and Positive Results for Crop and Cecal Samples of Broilers at Slaughter

			Crop Results		
Ceca Results			**Negative**	**Positive**	**Total**
	Negative	**% (Count)**	79.0 (854)	3.8 (41)	82.9 (895)
	Positive	**% (Count)**	12.9 (139)	4.3 (46)	17.1 (185)
	Total		91.9 (993)	8.1 (87)	100 (1080)

As illustrated in Table 4-17, total percentage of positive samples for crop and cecum were 8.1% and 17.1%, respectively. Similarly, total percentages of *Salmonella* negative samples were 91.9% for crops and 82.9% for ceca. In addition, 79.0% of both

crop and cecal samples of broilers were negative for *Salmonella* species, and 4.3% of both crop and cecal samples of broilers were positive for *Salmonella* species. Further, 3.8% of crop samples were positive for *Salmonella* species, but negative for *Salmonella* species in cecal samples. Similarly, 12.9% of the crop samples were negative for *Salmonella* species but positive for *Salmonella* in cecal samples.

CHAPTER 5

General Discussion

5.1 Findings of the study

5.1.1 Prevalence of *Salmonella* species in broiler chickens

In this study, cecal samples of broiler chickens were first processed by real time polymerase chain reaction (PCR) to identify *Salmonella* positive flock. Cecal samples from flocks that were negative by PCR were not processed further for *Salmonella* isolation. This PCR has been validated to be as sensitive as the traditional bacteriological culture method (Bohaychuk et al., 2007). In flocks that were positive for *Salmonella* (i.e., at least one cecal sample positive by PCR), crop and neck skin samples were processed for *Salmonella* isolation and further identification. Hence, both estimates of flock prevalence as well as within flock prevalence (i.e., sample level prevalence) of *Salmonella* species in broiler chickens at slaughter were available for analysis.

5.2.1.1 Flock prevalence of *Salmonella* species in broiler chickens

Of the 63 flocks, 36 had at least one cecal sample that was positive for *Salmonella* species detected by real-time PCR resulting in a flock prevalence of 57.1%. Cecal sample screening was the principle method used in this study to identify a positive flock as ceca are considered the primary site of *Salmonella* colonization in poultry (Barrow et al., 1988; Fanelli et al., 1971; Snoeyenbos et al., 1982). Furthermore, confirmation of *Salmonella* species in ceca is the most reliable indication that a chicken has been infected (Rigby & Pettit, 1978). One infected bird in a flock will make the flock positive for *Salmonella* species.

The flock prevalence of *Salmonella* species determined from cecal samples at slaughter was reported to be 50.0% in studies conducted by Boulianne et al (2004) and Arsenault et al. (2007) in Quebec, Canada, which is comparable to that observed in my study (57%). However, in the study by Boulianne et al. (2004), flock prevalence was much higher (87.5%) when carcass rinses from same birds were analyzed instead of cecal samples indicating an extraneous source of infection (e.g., in-plant contamination) in carcass rinses. As skin samples in my study were processed only from positive flocks, the flock prevalence from skin samples could not be estimated and it was not possible in this study to investigate the difference in flock prevalence of *Salmonella* species between skin and cecal samples.

In studies using *Salmonella* species isolated from litter or environmental samples from the farm, flock prevalence was 75% in a study conducted in early 1990s in Canada (Poppe et al., 1991), and 37% in a study conducted more recently in Ontario (Arsenault, 2005). In summary, flock prevalence appears to be dependent on the source of the samples used for identification.

Published reports indicated a huge variation in flock prevalence of *Salmonella* between countries ranging from 0.0% in Sweden (EFSA, 2007) to 100% in Thailand (Sasipreeyajan et al., 1996). Even within the European Union members, a huge variation in flock prevalence of *Salmonella* species has been reported with prevalence ranging from 0.0% in Sweden to 68.2% in Hungary (EFSA, 2007). However, these results are not comparable with this study or other studies because of the possible differences in sampling plans, types of sample cultured, and microbiological methods used to isolate the organisms. This lower prevalence might be related to the excellent *Salmonella* control

program in place for the entire food chain from "farm to fork" in several European countries including Sweden and Norway (Anonymous, 2004a; Anonymous, 2004b).

5.2.1.2 Within flock prevalence of *Salmonella* species in broiler chickens

Three different types of samples – namely crops, ceca, and neck skins – were processed in this study to estimate the within flock prevalence or sample level prevalence of *Salmonella* species of broiler chickens at slaughter.

5.2.1.2.1 Prevalence of *Salmonella* species in cecal samples

A sample level prevalence of 9.8% was observed in this study. Similarly, the sample level prevalence of *Salmonella* species for cecal samples among positive flocks was 17.2%. The sample level prevalence of *Salmonella* species in this study was in agreement with those results reported in a study conducted in Turkey (Carli et al., 1991). The prevalence of *Salmonella* species from cecal samples obtained by the Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) during abattoir surveillance in Canada were 13%, 16%, 16%, and 18% for 2002, 2003, 2004, and 2005, respectively (PHAC, 2007). These numbers, though comparable, are higher than that observed in study (9.8%). CIPARS (PHAC, 2007) is a national program, which receives samples from all 10 provinces, and sampling is conducted throughout the year. In contrast, this study was limited to a slaughterhouse in Alberta, and sampling was done mainly during the winter months of 2004to 2005 (November to April). Different geographic locations included in the National study, and the associated differences in farming practices as well as the different culture methodology used may have contributed to the differences noted between these studies.

5.2.1.2.2 Prevalence of *Salmonella* species in crop samples

In this study, sample level prevalence of *Salmonella* species isolated from crop within the positive flocks was 8.1%. However, crop samples collected from flocks in which cecal samples tested negative by PCR, were considered negative for *Salmonella* species and were not subjected to further microbiological analysis. The *Salmonella* prevalence recorded in crop samples in this study was higher than the prevalence (4.3%) reported for Ontario and Quebec by Chambers et al (1998). Higher prevalence in this study might be related to increased sensitivity of *Salmonella* isolation from positive flocks.

5.2.1.2.3 Prevalence of *Salmonella* species in neck skin samples

In this study a sample level prevalence of *Salmonella* species from skin samples was 53.9%. However, recent studies in North America have identified a lower prevalence of *Salmonella* species in broiler chickens at slaughter; 21.1% in Canada (CFIA, 2000) and 20.0% in the U.S. (USDA, 1996). However, those studies used carcass rinse for *Salmonella* culture in contrast to the enrichment culture of a skin sample used in this study. The recovery rate of *Salmonella* species would be higher when neck skin samples were cultured (Meekin et al, 1984; C. Poppe, personal communication, October 2004) rather than carcass rinses (Jogensen et al., 2002). This was attributed to the inability of carcass rinses to gather all the *Salmonella* species attached to the skin (Lillard, 1989). Furthermore, skin samples from flocks that tested cecal samples negative by PCR were not further subjected to microbiological analysis in this study.

CIPARS (PHAC, 2006) results for the year 2004 indicated that 17% of retail meat samples collected from Ontario and Quebec were positive for *Salmonella* species. Considering the likely reduction in bacterial count between processing and retail

(e.g., final rinse, refrigeration etc.), and the fact that only neck skin samples from cecal-PCR positive farms were cultured in this study, the overall prevalence of *Salmonella* detected in this study (53.9%), therefore seems higher in comparison with other North American studies (CFIA, 2000; USDA, 1996). However, *Salmonella* prevalence in broiler chickens at slaughter cultured from carcass rinses in several other countries was comparable to the results from this study (53.9%), including 60.9% in a previous study in Canada (Lammerding et al., 1988), 50.0% in Malaysia (Rusul et al., 1996), and 56.7% in Spain (Caraminana et al., 1997).

Prevalence of *Salmonella* in neck skin samples reported in certain European countries ranged between 0 to <0.1%(EFSA, 2006), which was significantly lower compared to the prevalence in the current study This lower prevalence might be related to the excellent *Salmonella* control program in place for the entire food chain from "farm to fork" in several European countries including Sweden and Norway (Anonymous, 2004a; Anonymous, 2004b). Furthermore, flock prevalence of *Salmonella* in these countries is extremely low, ranging from 0 to 0.1% (EFSA, 2007).

In addition, it was observed that *Salmonella* prevalence in crops and ceca were relatively lower compared to *Salmonella* prevalence on neck skin from the same flock. Hence, there might be the possibility of carcass contamination during processing. Cross-contamination of carcasses during processing, (e.g., defeathering, evisceration, chilling tank etc), is known to occur for several intestinal commensals or pathogens of chickens (Dougherty, 1976; Lillard, 1990; Morris & Wells, 1970). Such cross contamination could occur from a few infected birds in the same flock or even from flocks processed previously. In contrast, as the ceca or crops were collected from individual birds and their

contents were opened in the laboratory for culture using standard aseptic practices, it is unlikely that cecal or crop contents would subject to cross-contamination. However, cross-contamination of crops and ceca can occur during transportation and waiting time at plant in contaminated crates (Rigby et al., 1980a). Hence, cecal or crop prevalence of *Salmonella* probably reflects the true infection rate among birds, although skin contamination is likely to have more direct human health implications.

5.2.2 Risk factors for *Salmonella* species contamination in broiler chickens

In this study, information on several potential risk factors such as on farm-feed withdrawal time, transport time, wait time at plant, size of the farm, number of barns on farm, on-farm quality program, mortality percentage, age of the bird, environmental temperature, condemnation percentage at plant, and order of kill at plant was collected using a questionnaire. Information on age, mortality percentage, and number of barns was missing for several flocks; therefore, these factors were not included in the final analysis.

5.2.2.1 Main risk factors identified in this study

In this study, the total feed withdrawal time ranged from 9.7 hours to 17.7 hours, with a mean of 12.8 hours. Total feed withdrawal time was further subdivided into on-farm feed withdrawal time, transport time, and wait time. On-farm feed withdrawal time ranged from 1.8 hours to 15.8 hours, with a mean of 6.8 hours. Similarly, transport time varied from 0.3 to 8 hours (mean= 2.5 hours) and wait time in plant varied from 0.0 to 8.3 hours (mean= 3.4 hours). In this study, two flocks had more than 15 hours of on-farm feed withdrawal time, one flock had 8 hours of transport time.

Several studies have identified longer feed withdrawal time as a risk factor for *Salmonella* contamination of crop and ceca of broiler chickens at slaughter

(Hargis et al., 1995; Ramirez et al., 1997; Corrier et al., 1999). It was suggested that the increased occurrence of *Salmonella* in crop contents was associated with an increased tendency of broilers to consume contaminated litter during feed withdrawal (Corrier et al., 1999). Also, longer feed withdrawal time creates physical, chemical, and microbiological changes in the crops of broiler chickens which may decrease the natural resistance of the birds to crop colonization by *Enterobactericeae* including *Salmonella* (Hinton et al., 2000). In previous studies, feed withdrawal time was not broken down to on-farm feed withdrawal time, transport time and waiting time at plant. In this study, although the total feed withdrawal time was not associated with increased *Salmonella* contamination of crop, ceca, and neck skin of broiler chickens, longer wait time at plant was associated with increased *Salmonella* prevalence in crops and ceca. Birds that had to wait for longer period at plant, after already being off-feed on-farm and going through the stress of transport, are likely to eat litter while waiting at the plant, as suggested by Corrier et al (1999). This could have led to the higher prevalence of *Salmonella* in crop and ceca of birds that had a longer wait time at the plant. If longer wait time increases the likelihood of birds eating the litter, several birds are likely to get exposed to *Salmonella* from a few infected birds within their own flock. Additionally, they might acquire the infection by ingesting contaminated materials left in the trucks or transport crates from other infected flocks that had been transported previously. Transport trucks or crates have been known to be a significant source of *Salmonella* contamination for birds (Rigby et al., 1980).

In this study, longer transport time was associated with an increased prevalence of *Salmonella* species in neck skin samples of broiler chickens. The increase in prevalence

of *Salmonella* on skin might be related to the increased shedding of *Salmonella* associated with transport stress during the lengthy journey or the increased possibility of cross-contamination during transportation (ACMSF, 1996; FAO, 2005a). During transportation, birds are often stored in open crates that are placed on the top of each other, and feces can drop from an upper crate to a lower crate contaminating feathers and body surfaces of birds (FAO, 2005b). Movement during travel also increases the possibility of spreading *Salmonella* over the body surface, thereby increasing the recovery of *Salmonella* in neck skin (McMeekin et al., 1984; Jorgensen et al., 2000). Once *Salmonella* species attaches to body skin, it is difficult to eliminate during washing procedures at plant (Lillard, 1989). Increased shedding of *Salmonella* during transport could also increase the chances of birds eating contaminated litter during transport or during waiting time at the plant. However, why increased transport time was not associated with the increased prevalence of *Salmonella* in crops and ceca in this study is not known. Continuous movement during transport might reduce the tendency for birds to eat litter, thereby limiting their oral exposure to the pathogen, resulting in lower prevalence of *Salmonella* in crops and ceca. Similarly, birds with longer transport time had shorter wait time at the plant, thereby limiting their exposure to contaminated litter. Future studies could be designed to investigate these factors in more detail.

5.2.2.2 Other risk factors identified in this study

In addition to transport and wait time at plant, this study identified outdoor temperature and flock size as other risk factors for increased *Salmonella* contamination of broiler chickens at slaughter. Lower outdoor or environmental temperature was

associated with higher prevalence of *Salmonella* in ceca. The findings are in agreement with Australian (Soerjadi-Liem & Cumming, 1984) and Danish studies (Angen et al., 1996; Skov et al., 1999), which indicated a higher probability of *Salmonella* infection during cold and wet season. In this study, the prevalence of *Salmonella* was the highest in January and lowest in April for crops, ceca, and neck skins. The highest level of contamination during the month of January might have been associated with increased stress associated with transportation of birds at lower environmental temperatures, resulting in increased excretion of *Salmonella*. However, as this study did not encompass all seasons of the year, it is hard to make any definite conclusions about the seasonal influence in *Salmonella* shedding, and this needs to be addressed by future studies. In addition, larger flock size increased the risk of *Salmonella* contamination of the skin of broiler chickens. These findings are consistent with studies that evaluated flock size as a risk factor for *Salmonella* infection of the flock (Angen et al., 1996; FAO, 2005a). However, in another study, flock size was not identified as a risk factor for *Salmonella* contamination of the flock (Skov et al., 1999).

It is interesting to note that prevalence of the *Salmonella* on neck skins, but not in crops or ceca, was higher on samples collected on Wednesdays than on Mondays or Tuesdays. However, this research was not primarily designed to evaluate days of the week as a risk factor for *Salmonella* contamination. Therefore, it was not possible to confirm whether processing facilities were more contaminated with pathogen later during the week. This needs to be further investigated in the future.

5.2.3 Distribution of serotypes and phagetypes of *Salmonella* species in broiler chicken

The most frequently isolated serovar in this study from crop, cecal, and neck skin samples was Hadar followed by Heidelberg This finding is similar to observations reported by Chambers et al. (1998). However, during 2005 abattoir surveillance in Canada, the top three serovars from chickens were Heidelberg, Typhimurium and Enteritidis, accounting for 29%, 5%, and 3.5% respectively (PHAC, 2007). Furthermore, Heidelberg is one of the top five serovars isolated from human cases in Canada and the U.S. (PHAC, 2007).

5.3 Significance of the study

This study provided valuable information for the industry and scientific community on the prevalence of *Salmonella* species in crops, ceca, and neck skins of broiler chickens at slaughter in Alberta. To the best of the author's knowledge, this is the first study to investigate within-flock prevalence of *Salmonella* species from three different samples from the same flock, and to examine their associations with potential risk factors.

The principle research question tested in this study was to evaluate the effects of feed withdrawal time on prevalence of *Salmonella* in various tissues, namely crops, ceca, and neck skin, for broiler chickens at slaughter. In this study, feed withdrawal time was divided into different phases, such as on-farm feed withdrawal time, transport time, and waiting time at plant. Their associations with the prevalence of *Salmonella* on crops, ceca, and skins of broiler chickens were further analyzed. Previous studies evaluating the association between feed withdrawal time and prevalence of *Salmonella* either in crops or ceca had not divided overall feed withdrawal time into different stages. Furthermore,

those studies, with the exception of Corrier et al (1999a), were conducted in experimental setting (Ramirez et al., 1997; Hargis et al., 1995: Hinton et al., 2000). The results of this study, conducted in a commercial setting, are directly applicable to risk management programs that could be developed to minimize contamination of chicken meat with *Salmonella* species. Furthermore, this study identified the importance of dividing the total feed withdrawal times into different stages while studying its association with the *Salmonella* contamination in chicken tissues or carcasses at slaughter. Considering the importance of these findings in developing future risk management programs, it would be important to further validate the findings of this study in future research.

5.4 Limitation of the study

The sampling period of the present study was limited between November and April rather than the whole year. As a result, it was not possible to compare the impact of seasonal differences on prevalence of *Salmonella*. Furthermore, it was not feasible to randomize the sampling days of the week due to constraints associated with laboratory processing of the samples. The sampling time at plant was also limited to morning hours due to the operating hours of the processing plants. Therefore, sampling days were Mondays, Tuesdays, and Wednesdays only, and sampling time was scheduled for 6 am, 7am, 8 am, and 10 am. The study showed that birds slaughtered on Wednesday were more likely to be positive for *Salmonella* on skin sample, suggesting a possible contamination built-up during the other working days of the week. However, this could not be confirmed within the limits of this study.

Information about on-farm practices was collected through a questionnaire based on data provided in the "*Flock Information Reporting Form*" by farmers to processors. Farmers could not be contacted to verify the information because of confidentiality requirements. Although, the information gathered in the questionnaire was randomly verified with the original Flock Information Reporting Form, these data originated in rather a passive manner. This might have compromised the quality of data available for analysis.

In addition, due to cost, all samples from 63 flocks were not processed. Preliminary screening of cecal samples by PCR was used to identify positive flocks and only the samples from positive flocks were further processed. Cecal samples were used for screening as it has been identified in other studies that the isolation of *Salmonella* from the ceca was the most reliable indication that the chicken or the farm was infected (Rigby and Petit, 1978). Further, the objective was to explore the stress of longer feed withdrawal time on the prevalence of *Salmonella* and its impact on carcasses if *Salmonella* was present in the flock. However, it is possible that some of the positive flocks or samples might have been missed during the preliminary screening affecting overall results.

All the *Salmonella* isolates were first analyzed by PFGE. Only a representative *Salmonella* isolate showing less than 95% homology during PFGE in each flock was further characterized by serotyping and phagetyping. It was hypothesized that isolates within a serotype/phagetype will be more than 95% homologous on PFGE. The idea behind it was to reduce the number of *Salmonella* isolates for serotyping and

phagetyping, ultimately reducing cost and labor. In doing so, we might have missed some serotypes that might have more than 95% homology, but were actually different.

CHAPTER 6

Conclusions and Recommendations

In this study, the flock prevalence of *Salmonella* species of broiler chickens in Alberta was estimated to be 57.1%. The overall sample level prevalence of *Salmonella* species in broiler chickens for cecal contents was 9.8%. Sample level prevalence of *Salmonella* species in broiler chickens among positive flocks was 8.1%, 17.2%, and 53.9% for crop, cecal, and neck skin samples, respectively. *Salmonella* Hadar and *S.* Heidelberg were the most common serovars identified from crop, cecal, and neck skin samples, indicating they are the most prevalent serovars in the broiler chickens.

In addition, the waiting time at the slaughter plant and travel time from farm to the slaughter plant were found to be risk factors for *Salmonella* contamination in broiler chickens. Longer waiting time at plant was associated with an increase in the recovery of *Salmonella* species from crops and ceca. Similarly, longer travel time was associated with an increase in the *Salmonella* contamination on neck skins of broiler chickens. In addition, lower environmental temperature and larger farm size were also identified as risk factors for *Salmonella* contamination. However, total feed withdrawal time, which included feed withdrawal time on farm, travel time and waiting time at plant was not found to be a significant factor for *Salmonella* contamination of crops, ceca, and neck skins in broiler chickens at slaughter. Other factors, such as on-farm quality program, condemnation percentage in plant, and kill order in plant, were not found to be significantly associated with *Salmonella* contamination of broiler chickens at slaughter.

The isolation rate of *Salmonella* species from neck skin samples of the broiler chickens at slaughter was also significantly associated with the day of the week that the

samples were collected. The contamination rate was higher for Wednesdays compared to Mondays and Tuesdays. However, the sampling day of the week seemed to have no influence on the *Salmonella* prevalence in crop and cecal samples. This association may be related to a potential build up of contamination over the working days of the week in processing plant facilities, especially where cross contamination between carcasses could occur, in scalding or cooling tanks, for example. However, this study was not designed to evaluate the effect of the day of sampling in *Salmonella* prevalence, and hence the possibility of contamination build up at the plant cannot be answered by this study.

Results also suggested significant differences in prevalence of *Salmonella* in crops, cecal, and neck skin samples, depending on sampling month. The highest prevalence of *Salmonella* was observed in January for all three samples and the lowest was observed in April. As the sampling was done from November to April only rather than a whole year the seasonal influence is difficult to interpret due to potential biases. However, it might be possible that the association could have been related to the increased stress associated with transportation of the birds at lower environmental temperature, which might have increased the excretion of *Salmonella*.

This study confirmed the research question that longer transport time and wait time at plant increase *Salmonella* contamination of broiler chickens at slaughter. Therefore, the broiler industry, including processing plants, should work together to reduce the travel time and wait time at plant. Reducing transport time is often difficult due to the location of slaughterhouses. However, it could be planned in such a way that flocks with longer transport times be slaughtered with minimum wait time at the plant.

Such a simple modification in kill-order could potentially minimize *Salmonella* species contamination in broiler carcasses.

Future research on feed withdrawal time and *Salmonella* contamination should focus on investigating the association within larger geographic areas, different slaughter plants, or birds raised under different management practices. Furthermore, influence of sampling day and the month of *Salmonella* contamination of broiler chickens should be explored in order to identify whether there are interactions between feed withdrawal time and season or day of slaughter that significantly influence the outcome. A round-the-year study will be required for such investigations. In this study, the feed withdrawal time was estimated based on the flock sheets. It would be desirable to verify the feed withdrawal time on farm directly from producers.

Reducing stress related to longer transport time would lead to decreased *Salmonella* contamination on skin of broiler chickens. Furthermore, reduction in the waiting time at plant before they are processed will reduce the prevalence of *Salmonella* in crops and ceca, which in turn will reduce the *Salmonella* prevalence on carcasses due to cross-contamination in plant during the evisceration process.

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Appendix 1

Research Questionnaire

SALMONELLA PROJECT

The Effect of Feed Withdrawal Time on *Salmonella* Contamination of Crops, Ceca and Carcasses of Broilers at Slaughter

Instructions: Please mark your choices in the boxes provided with an "X" wherever applicable.
Please Print

General

Edmonton Plant ☐

Calgary Plant ☐

Flock Number:_____ Lot Number:_____

Date Sampled ..... M | D | Y

Sampling Start Time..... TIME | | AM___ PM___

Sampling End Time..... TIME | | AM___ PM___

Information Provided By: _____

A. Basic Flock Elements

Was the On-Farm Food Safety Assurance Program implemented during this cycle?.....Yes ☐..... No ☐

Categories / Sex: Pullets ☐ Cockerels ☐ Roasters ☐ Non-sexed ☐

B. Diseases and Treatments During Grow-Out Period

Was there any disease outbreak during the grow-out period?.....Yes ☐..... No ☐

(If yes, please list all diseases/syndromes and treatment administered during grow-out)

Name of Disease or Syndrome	Medications (including dosage)	Safe Marketing Date as per Recommended Withdrawal Time (if any)	Method Administered (water/feed)	First Treatment Date	Last Treatment Date

Note: All answers will be held in strict confidence. Only summary information will be reported.

C. Feed and Withdrawal

1. Any feed medication requiring withdrawal? ..... Yes ☐ ..... No ☐
   (If yes, please fill out the spaces provided below)

   Antibiotic _____ Withdrawal period _____ Safe Marketing Date | M | D | Y |

   Coccidiostat _____ Withdrawal period _____ Safe Marketing Date | M | D | Y |

2. Actual beginning of catching/loading ..... | M | D | Y | | TIME | | AM__ PM__ |
3. Actual ending of catching/loading ..... | M | D | Y | | TIME | | AM__ PM__ |
4. Time feeders were raised ..... | M | D | Y | | TIME | | AM__ PM__ |
5. Time of last access to water ..... | M | D | Y | | TIME | | AM__ PM__ |
6. Was the feed supply disrupted within the last 48 hours? ..... Yes ☐ ..... No ☐
7. Transport Time ..... _____ Hours
8. Waiting time in the Plant ..... _____ Hours
9. Exact Kill Time ..... | M | D | Y | | TIME | | AM__ PM__ |

D. Grow-Out Data

1. Age of Birds ..... _____ Days
2. Size of Flock (total number of birds) ..... _____
3. Total Number of Barns ..... _____

Grow-Out Barn No.	Placement Date	Number of Birds Placed (include extras)	Estimated Mortality Rate (%)	Estimated Live KG Per Bird	No. of Birds Shipped	Grow-Out Area (square feet/bird)

E. Plant Information

1. Number of Birds in Lot ..... _____
2. Order of Kill ..... _____
3. Condemnation Rate ..... _____

Note: All answers will be held in strict confidence. Only summary information will be reported.

Appendix 2

Flock Information Reporting Form

Flock Information Reporting Form

Producer/Enterprise Name: _____ Producer Code: _____ Quota #: _____

Flock #: _____ Barn #: _____ Floor #: _____ Species: _____ Category/Sex: _____

Age of Birds being Shipped: _____ # Birds Placed: _____ Estimated Mortality Rate (%): _____

Estimated # Birds Shipped: _____ Estimated Live Kg per Bird: _____ Grow-out Density: _____

Section A (Hatchery and Vaccine Information)

Vaccines and treatment (include withdrawal period) at the hatchery level as indicated by the hatchery	Vaccination during growing/production period and specify method (water, air, injection)
Date:_____	Date:_____
Date:_____	Date:_____
Date:_____	Date:_____

Section B (Diseases and Treatments During the Grow-Out Period)

Were any diseases requiring medication observed during grow-out? ..... ☐ No ☐ Yes (list all in table below)

Name of Disease or Syndrome	Medication Used	Dosage	Method Administered (water/feed)	First treatment date	Last treatment date	Flock recovered (grower's initials)	Safe marketing date as per recommended withdrawal time (if any)

Section C (Feed and Feed Withdrawal)

	M	D	Time AM PM
Planned catching/loading time:			
Actual beginning of catching/loading: ...			
Planned processing time:			
Time of last access to water:			

Was the feed supply disrupted in the last 48 hours? .... ☐Yes ☐No

Were any preventative medications requiring a withdrawal period used in the last 14 days? ☐Yes ☐No .... **If yes:**

Product Name	Withdrawal Period	Safe Marketing Date

	M	D	Floor #1 Time AM PM	Floor #2 Time AM PM	Floor #3 Time AM PM
Time feed was no longer accessible:					

Was the feed withdrawal time provided by the processor: .... ☐Yes ☐No .... **If yes:**

M	D	Time AM PM

Provide any additional comments on flock condition during the grow-out period and/or catching process on a separate sheet of paper if desired.

I confirm that, to the best of my knowledge, the information contained on this flock information reporting form is accurate and complete and that any diseases that were diagnosed in the flock as a result of laboratory tests and/or readily observable clinical signs have been identified and reported on this form.

Producer's Signature: _____

Note: This information is confidential between the producer and the processor.

Appendix 3

Salmonella Isolation and Identification

For processing in the laboratory, each sample of crop and ceca were transferred into a sterile petri dish and surfaces were disinfected with 70 % ethanol alcohol in order to prevent cross contamination.

After the incision with sterile surgical blade approximately a 2 g of cecal content and 1 g of crop content were inoculated to 18 mL and 9 mL of pre-enrichment broth, Buffered Peptone Water (BPW) (Becton and Dickinson Company) respectively. Ten to twenty g of neck skin samples were pre-enriched in BPW (BD) in a 1:9 w/v proportion.

After the BPW incubation at 35°C for 20-24 hr, 0.1 mL of BPW was inoculated into 10 mL of the selective enrichment broth, Rappaport-Vassiliadis Broth (RV) (EMD Chemicals, Darmstadt, Germany). At the same time 1 mL of BPW was inoculated into Tetrathionate Broth (TT) (BD), to which 0.2 mL of iodine solution had been added just prior to use. Both RV and TT tubes were then incubated at 42°C for 22-24 hr.

After incubation and vortexing, 0.15 mL from each of the RV and TT were pooled first and then screened for *Salmonella* using real-time PCR. No further testing was performed on real-time PCR negative samples and these were accepted as a negative result. Real-time PCR positive samples were confirmed by culture. These results were considered as final results.

For culture, 10 µL of each RV broth and TT broth were streaked onto xylose lysine tergitol 4 (XLT4) (BD) and Rambach (RAM) (EMD) selective agar plates and incubated at 35°C. Plates were read after 18-24 hr and 48 h of incubation. Suspected-*Salmonella* colonies from XLT4 and Rambach plates were screened using Triple Sugar

Iron Agar slants (TSI), Urea agar slants and Lysine Iron Agar slants (LIA), and plated to a ¼ blood agar plate (BAP) and ¼ MacConkey plate (MAC) to check for purity.

One *Salmonella*- suspected isolate was then tested with *Salmonella* Poly O and Poly O1 antisera agglutination (Denka Seiken Co. Ltd. Japan). Isolates demonstrating a positive agglutination reaction and characteristic biochemical reactions were considered to be presumptive *Salmonella.* Two additional *Salmonella* isolates per sample (based on typical colonial morphology) were chosen and all three isolates were frozen at –70°C.

Appendix 4

Pulse-Field Gel Electrophoresis (PFGE) Analysis

Fresh bacterial cultures grown on blood agar plates no longer than 24 hr were used for making plugs. *Salmonella* serotype Newport am01144 was used as a reference strain for profile analysis. Bacterial isolates were swabbed into ~ 5 mL of cell suspension buffer (100 mM Tris; 100 mM EDTA) and were standardized to an optical density of 1.3-1.4 using a Genesys 20 spectrophotometer (Thermo Spectronic, Rochester, NY). Five hundred microliters of suspension was transferred to a 1.5 mL microfuge tube and Proteinase K was added to a final concentration of 1mg/mL. After gentle mixing of bacteria/PK mixture, 500 µL of 1% molten SeaKem Gold agarose containing 1% sodium dodecyl sulphate were added to each vial followed by mixing.

Plug molds (Bio-Rad, Mississauga, ON) were filled immediately and allowed to solidify at room temperature before the plugs were placed into 1.5 mL of cell lysis buffer (Tris-HCl: 50 mM; EDTA: 50 mM; sarcosyl: 1%; PK: 0.5 mg/mL). After incubation of plug molds for 2 hr in a 54°C waterbath, a 2 mm slice from each plug was placed in a fresh microfuge tube and the remainders of the plugs were stored at 4°C. Each slice was washed twice with 750 µL of sterile, Type 1 water for 15 min at room temperature (RT) followed by 4 washes of TE buffer (10 mM Tris; 1 mM EDTA). The final TE wash was removed and replaced with 750 µL of reaction buffer (0.05M Tris-HCl; 0.01M Magnesium chloride; 0.05M Sodium Chloride) and then incubated for 15 at RT. After that reaction buffer was removed and replaced with 200 µL of 1X REact 2 buffer (Invitrogen, Mississauga, ON) followed by incubation at 37°C for 15 min. REact 2 buffer was removed and 200 µL of 1X REact buffer containing 0.3U/µL of *Xba*1 restriction

enzyme (Invitrogen) were added followed by incubation at 37°C for 2 hr. The slices were allowed to sit at RT for 5 min after enzyme mixture was removed and replaced with 500 µL of 0.5X TBE (0.05M Tris-HCl; 0.01M Magnesium chloride; 0.05M Sodium Chloride).

Gel slices were run in a 1% SeaKem Gold agarose gel in a CHEF-DRIII PFGE system (Bio-Rad) using 0.5X TBE at 14°C as running buffer and Thiourea was added to running buffer to a final concentration of 50µM for bacterial strains that smeared during routine PFGE analysis. Following electrophoresis, the gels were stained at RT in ~ 400 mL of Type 1 water containing 1.0 µg/mL ethidium bromide for 30 min on a gelsurfer mixer. After staining, excess ethidium bromide was removed by placing the gel under running tap water for ~ 5 min. Digital images of the gel were captured using a UV tranillumination, Kodak 1D Image Analysis Software and a DC290 Kodak camera. Gel images were downloaded into BioNumerics software (Applied Maths, Lint-Martens-Latem, Belgium) and analyzed using a Dice coefficient of 0.5% and a tolerance of 1.0%.